LISTERIA MONOCYTOGENES PATHOGENESIS:
PART I: THE ROLE OF FLAGELLA MEDIATED MOTILITY
PART II: THE FUNCTION OF THE METALLOPROTEASE PROPEPTIDE

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LISTERIA MONOCYTOGENES PATHOGENESIS:
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Listeria monocytogenes is a food borne, intracellular bacterial pathogen. Successful infection requires completion of two steps: penetration of the intestinal epithelium and escape from the phagocytic vacuole. This dissertation examines the role of flagella mediated motility in host cell invasion, and the function of the metalloprotease (Mpl) propeptide in mediating the maturation of a bacterial phospholipase required for efficient escape from the phagocytic vacuole.

We examined the contribution of flagella to *L. monocytogenes* pathogenesis. We observed that flagella mediated motility enhance the bacterial rate of invasion. To determine if flagella are adhesins, we performed adhesion and invasion assays with flagellated motile and non-motile bacteria, and non-flagellated bacteria. Flagellated but non-motile bacteria did not adhere to or invade human epithelial cells more efficiently than non-flagellated bacteria. These results indicate that flagella do not function as adhesins to host cells. Instead, motility is important for host cell invasion. Moreover, *in vivo* motile bacteria out competed non-motile bacteria in the colonization of the mouse intestines and liver at early time points after oral infection, suggesting that flagella-mediated motility enhances *L. monocytogenes* infectivity soon after bacterial ingestion.

Mpl regulates the activity and compartmentalization of a bacterial phospholipase C (PC-PLC). Mpl is secreted as an inactive proprotein. In related proteases, the propeptide can serve as a folding catalyst (either *in cis* or *in trans*), influence protein compartmentalization, participate in intracellular trafficking, or decrease folding kinetics. We investigated the role of the Mpl propeptide by monitoring the behavior of Mpl synthesized in absence of its propeptide (MplΔpro) and of two Mpl mutants with unstable propeptides. All three mutants mediated PC-PLC activation *in vitro* but were not functional in infected cells. This defect was not rescued by providing the propeptide *in trans* to the *mpl*Δpro mutant. We also determined that PC-PLC co-purified with wild-type Mpl, MplΔpro, and the Mpl propeptide indicating that the propeptide is not required for Mpl / PC-PLC interaction. However, the mutant Mpl species were aberrantly secreted in the cytosol of infected cells. These data indicate that the propeptide of Mpl maintains Mpl bacteria-associated, and that localization is essential to Mpl function during infection.

BIOGRAPHICAL SKETCH

Heather O'Neil received her Bachelors of Science in Biology from Tufts University in Medford, MA in 1996. Later she completed a post-baccalaureate year at Mills College in Oakland, CA. Following that she worked for The National Food Laboratory, Inc. in Dublin, CA before deciding to pursue a Ph.D. She chose to attend Cornell University and received a USDA National Needs Fellowship (Food Safety) for her first three years of study, starting in August of 2002. After completion of her degree she intends to return to industry as a scientist in the biotechnology sector.

For Jeff and Henry

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I would like to thank my friends and family for their constant support throughout this endeavor. Thank you to my parents for always assuming that I would succeed, which made reaching my goal seem not only possible but inevitable. Thank you to my friends, especially those I made during graduate school. They were always there to sympathize over the difficult times, rejoice in the small triumphs and keep me focused on the ultimate goal. Most of all I want to thank my husband. His unwavering encouragement, constant support and tolerance of my difficult schedule were essential to my completion of this degree. Finally, thank you to my son Henry. Knowing that finishing this degree would impact my ability to provide for him and be a role model in his eyes was the best reason I had for coming to work each day.

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CHAPTER 1

INTRODUCTION

Part I. Background

1. History

The genus *Listeria* consists of six species of gram positive bacilli, two of these species are pathogenic (189). *L. seeligeri* is primarily a sheep pathogen. *L. monocytogenes* is a pathogen of humans and a wide variety of animals and causes the food borne disease listeriosis. It is distributed ubiquitously in nature as a soil bacterium of decaying vegetation and in the fecal flora of many animals (185). *L. monocytogenes* is able to grow in a wide variety of environments, importantly those that are also considered to be food preservatives (173).

Listeriosis was first described in a colony of laboratory rabbits and guinea pigs and almost concurrently as a deadly infection of wild gerbils in South Africa (148, 162). The epidemiology of listeriosis as a food borne disease was determined after an outbreak among hospital patients in Boston and after contaminated coleslaw sickened people in the Maritime Provinces of Canada (90, 180). Listeriosis persists as a public health issue. The Center for Disease Control estimates 2,500 cases of listeriosis annually in the United States resulting in 500 deaths (27). Due to the high mortality rate the Food and Drug Administration maintains a zero-tolerance policy for contamination of food with *L. monocytogenes* (57). The number of cases of listeriosis has declined since preventative measures were mandated; however there was no decrease in the incidence of listeriosis from the 2004-2006 data to 2007. The incidence level is not currently on track to meet the targets for the Healthy People 2010 national objectives (214). Part of this failure is due to the continued problem of large multi-state outbreaks (78). It is estimated that up to 2.4 billion dollars is spent annually on food safety measures to control *L. monocytogenes* contamination (93).

2. Listeriosis

L. monocytogenes is a facultative intracellular pathogen that can cross three separate barriers in the body: the intestinal barrier, the blood brain barrier and the placental barrier (50, 56, 210). *L. monocytogenes* is predominantly a pathogen of immune compromised individuals causing septicemia, meningitis and encephalitis. 30% of cases are in pregnant women usually causing a flu-like illness. Infection of the fetus can lead to abortion or listeriosis of the newborn. There is mounting evidence that *L. monocytogenes* can cause gastroenteritis in healthy adults if high doses of bacteria are ingested (3, 38). It is currently unclear what bacterial and host factors are involved in the infection of healthy adults.

As a food borne disease *L. monocytogenes* must first survive the highly acidic stomach then cross the intestinal barrier (Figure 1.1). The infectious dose and incubation time is unknown and most likely varies with strain and host factors (28). Once bacteria have successfully invaded the intestine they rapidly translocate to the liver, spleen and mesenteric lymph nodes via the lymph or blood. Bacteria in the liver are killed by neutrophils which lyse infected hepatocytes and clear extracellular bacteria (32, 80) Bacteria that survive begin increasing in number. Greater than 90% of the bacteria in an infected liver can be found in hepatocytes (35). Infected hepatocytes form microabscesses, release chemoattractants for neutrophils, and undergo apoptosis (174). Complete clearance of the bacteria from organs involves a response by CD8+ lymphocytes (164). Uncontrolled bacterial growth in the organs, more common in immune compromised hosts, leads to severe disease. *L. monocytogenes* has a tropism for the central nervous system as well as the gravid uterus, although the reasons for these tropisms are not known. Invasion of the brain occurs via circulating parasitized phagocytes (49, 51). This is a likely route for infection of the placenta; however

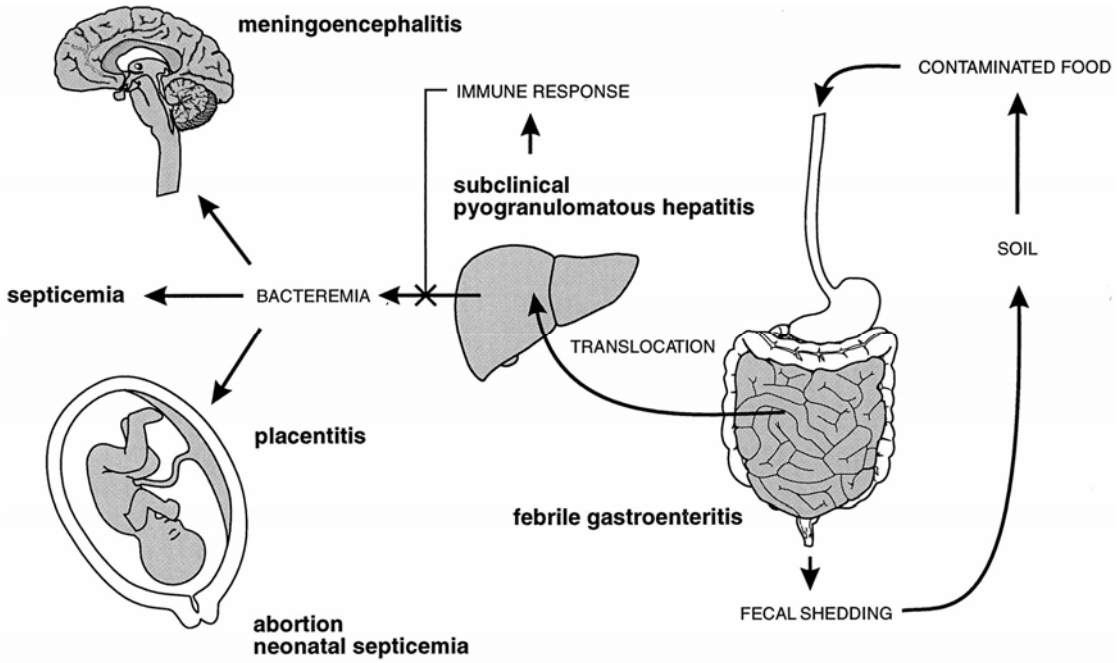

Figure 1.1. The pathophysiology of *Listeria* infection.

From Vazquez-Boland *et al*. 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. 14:584 - 640.

current pregnant animal models use an I.V. route of infection making it difficult to determine how initial seeding of the placenta occurs. Regardless, cell-to-cell spread appears to play an important role in infection of the fetus (4, 5). The placenta provides a protective niche for bacterial replication and results in the reseeding of other maternal organs (6).

3. Intracellular Life

L. monocytogenes can enter into a wide variety of host cells both phagocytic and non-professionally phagocytic. The different stages of *L. monocytogenes* intracellular life and cell to cell spread were beautifully elucidated using microscopy (146, 206) (Figure 1.2). Entry into the host cell is triggered by bacterial ligands and results in the bacterium being trapped in a membrane bound vacuole referred to as the primary vacuole (65, 86). The bacterium must escape from this vacuole to perpetuate infection (65, 66, 165). The vacuole acidifies and inhibition of vacuolar proton ATPases prevents escape (9, 33). The hemolysin, listeriolysin O (LLO) and phospholipases (PLCs) all play a part in mediating bacterial escape. Once in the cytoplasm of the host cell bacterial replication begins (86, 165).

Cytoplasmic bacteria are surrounded by a cloud of host cell actin that polymerizes into a long, cross linked tail. Intracellular movement and the formation of actin tails can be halted by the addition of Cytochalasin D, an inhibitor of actin polymerization (146, 206). Actin tail formation requires the expression of the bacterial surface protein ActA (17, 45, 109). ActA functions as an actin nucleator and is sufficient for cytosolic movement of ActA coated beads (24, 63, 163, 220) .

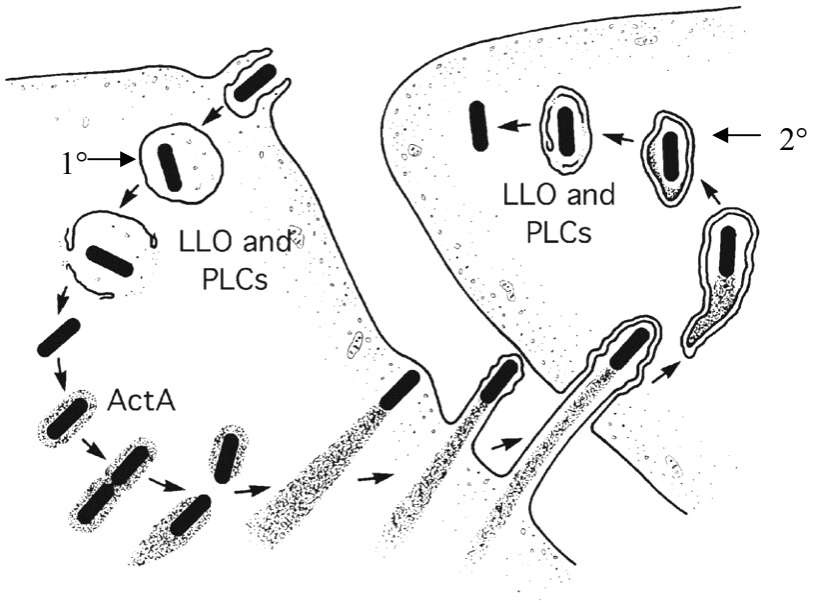

Figure 1.2. Stages in the entry, growth, movement and spread of Listeria from one macrophage to another. Vacuole formed upon initial host cell invasion is called the primary (1°) vacuole. Vacuole formed upon cell to cell spread is called the secondary (2°) or spreading vacuole.

Adapted from Tilney, L.G., and D.A. Portnoy. 1989. Actin filaments and the growth, movement and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. J. Cell Biol. 109:1597 - 1608.

The kinetics of intracellular spread was described using host cells expressing GFP-actin (172). A bacterium invades neighboring host cells by pushing against the plasma membrane of the donor cell and forming protrusions that invaginate into the accepting host cell. The bacterium then shows rapid fitful movement until the donor cell membrane closes about 15 minutes later. Bacterial movement ceases, presumably due to ATP depletion. The protrusion morphology does not change for another 20 minutes until the accepting cell membrane snaps shut, forming a spherical vacuole around the bacterium. This secondary vacuole is double walled, as the bacteria is enclosed in both the donor cell membrane as well as the acceptor cell membrane, and is permeabilized about 5 minutes later.

L. monocytogenes intracellular lifestyle of host cell invasion and cell to cell spread without leaving the intracellular milieu is a strategy that allows it to avoid the host humoral immune system. In fact, immunity is almost entirely cell mediated (164). While the specifics of the *L. monocytogenes* virulence factors are unique, this tactic is not. For example, *Shigella flexneri* is an intracellular bacteria that also invades host cells, escapes from the phagosome then moves intracellularly by polymerizing host cell actin (79). The use of host cell actin for movement is also seen in *Rickettsia* species although the morphology of the actin tails is different from *L. monocytogenes* and *S. flexneri* (79). *Burkholderia pseudomallei* is a gram negative bacterium that is also found intracellularly and escapes from the phagosome (79).

4. Regulation of virulence factors by PrfA and sigmaB

Successful invasion, escape from the vacuole, intracellular movement and cell to cell spread requires a variety of bacterial factors. Ten of these virulence factors have been definitively shown by transcriptome profiling and mechanistic studies to be under the

control of a regulatory protein called Positive Regulatory Factor A (PrfA) (Table 1.1) (144, 188). PrfA was originally identified due to its role in transcribing the gene for the first known *L. monocytogenes* virulence factor, the hemolysin listeriolysin O (LLO) (122). PrfA is required for virulence. Mutants without PrfA expression have reduced virulence gene expression resulting in the inability to replicate in host cytoplasm, the elimination of cell to cell spread, and avirulence in tissue culture and animal models (29, 62, 122, 138).

PrfA is a transcriptional activator that recognizes a DNA-binding site upstream from the controlled promoter (14, 226). The recognition sequence is a 14 base pair palindrome with dyad symmetry called the "PrfA box". It consists of 7 invariant nucleotides and is only tolerant of 1-2 mismatches among the other nucleotides (188). The varying affinity of PrfA for its target promoters is one way of regulating virulence genes. Promoters with perfect symmetry are more strongly expressed than promoters with mismatches (212). Palindrome sequence is not the only factor in promoter strength, as substitution of strong PrfA promoters for weaker PrfA promoters does not always cause increased transcription of reporter fusions (221). Many virulence factors also have additional promoters entirely independent of PrfA. Some genes, such as *actA*, *hly* and *inlAB*, contain 5' untranslated regions that affect mRNA translation (190, 200, 222).

PrfA is related to the regulator cAMP Receptor Protein (Crp), also known as the Catabolite gene Activator Protein (Cap), although the sequence similarity is low (114). PrfA and Crp have several similar structural features such as a Beta-roll, an alpha helical region, and a C-terminal domain with a DNA-binding helix-turn-helix motif.

Table 1.1. PrfA regulated virulence factors

Gene	**Protein function**
hly	Listeriolysin O
mpl	Metalloprotease
acta	Actin-assembly inducing protein
plcB	Phospholipase C
plcA	Phosatidylinositol-specific phospholipase C
inlA	Internalin A
inlB	Internalin B
inlC	Internalin C
hpt	Hexose phosphate transport protein
prfA	Positive regulatory factor A

PrfA does not bind cyclic nucleotides but is probably under allosteric control (15, 54, 171, 211). Studies of hyperactive PrfA mutants suggest that allosteric activation by an unknown cofactor changes PrfA from its weakly active native form to a highly active form (171, 211).

Several of the virulence genes under PrfA control are clustered together in a region called the *Listeria* pathogenicity island-1 (LIPI-1) (Figure 1.3). LIPI-1 includes the genes for listeriolysin O, *hly*, the metalloprotease, *mpl*, the actin polymerization protein, *actA*, the broad range phospholipase C, *plcB*, the phosphinositol specific phospholipase, *plcA*, and *prfA* itself. Transcriptome analysis has shown up to 145 additional sequences that show some level of differential expression associated with PrfA (133, 144). Some of these transcriptional changes are probably due to the effects of the alternative sigma factor sigmaB (discussed below).

There are multiple promoters for *prfA* (Figure 1.3). Promoters P1 and P2, immediately upstream of *prfA*, provide a constant low level of PrfA. This level of PrfA is present outside of host cells and at the beginning of infection and is sufficient to bind high affinity PrfA promoters and enable escape from the primary vacuole but does not support cell to cell spread (61, 62). The translation of PrfA transcripts from P1 is thermoregulated. The 5' untranslated region has secondary structure that obstructs the ribosomal binding site at lower temperatures, but melts at higher temperatures such as host body temperature (96, 121). This provides a sudden pool of transcripts ready to be translated when the bacteria enter the host. Once in the host cytoplasm PrfA synthesis increases and can now bind lower affinity promoters. PrfA is also transcribed from the virulence gene *plcA* promoter. This provides a positive feedback loop when PrfA activity and levels increase and virulence gene expression is

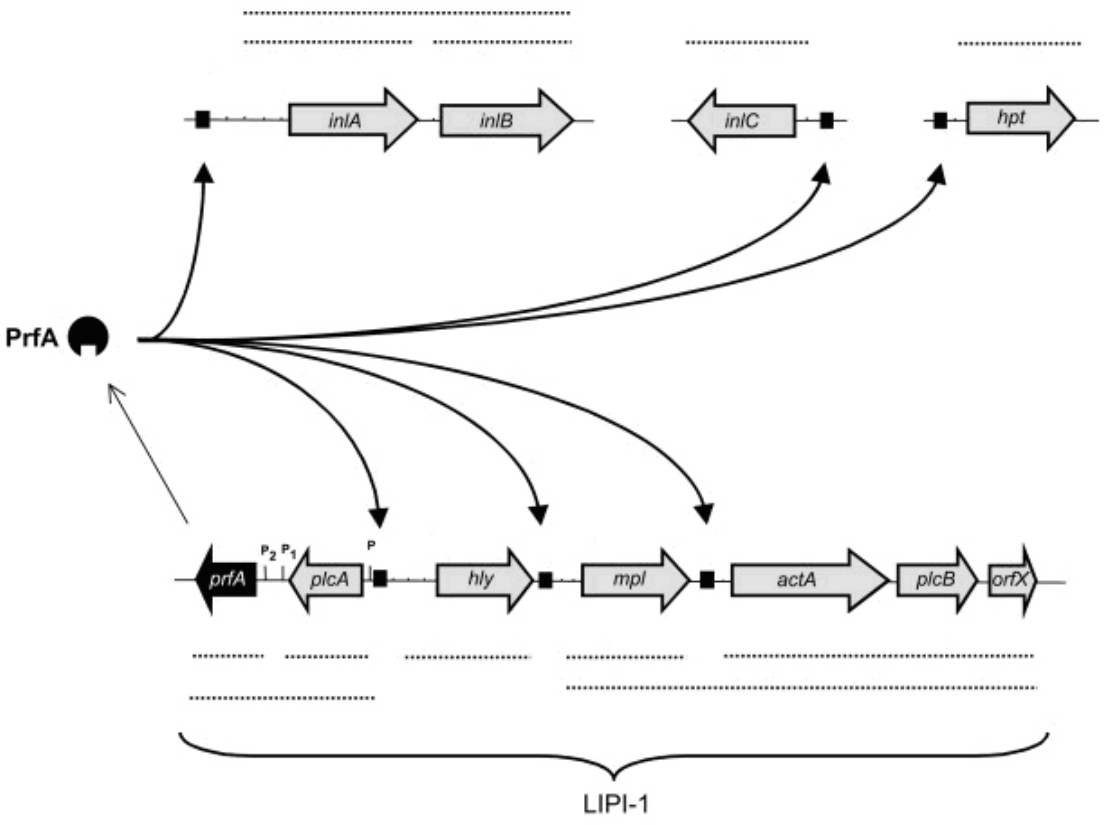

Figure 1.3. The core PrfA virulon of *L. monocytogenes*. Physical and transcriptional organization of *Listeria* pathogenicity island-1 (LIPI-1) genes and the three other PrfA-regulated loci of the *L. monocytogenes* genome: the *inlAB* operon, and the *inlC* and *hpt* monocistrons; genes pointing to the right are on the positive strand. PrfA-boxes are indicated by black squares, known promoters and transcripts are indicated by "P" and dotted lines, respectively.

Adapted from Scortti *et al.* 2007. The PrfA virulence regulon. Microbes. Infect. 9:1196 – 1206.

induced (29, 138). Loss of read-through transcription from *plcA* decreases virulence (26).

There is some evidence that during exponential growth *in vitro L. monocytogenes* produces a diffusible autorepressor that decreases PrfA activity without affecting transcription (55). Repression of PrfA activity is also seen when bacteria are grown on some carbohydrate sources (143).

The *L. monocytogenes* alternative sigma factor, sigmaB, also has a role in virulence. The association of RNA polymerase with DNA can be altered by sigma factors. Different sigma factors allow the core polymerase to recognize new target genes for transcription (87). SigmaB is involved in coordinating the response of *L. monocytogenes* to acid, carbon starvation, osmotic, temperature, and oxidative stresses (208).

There is a complex relationship between the *L. monocytogenes* stress responses and virulence. Multiple PrfA regulated virulence genes also have putative sigmaB dependent promoters (144). For example, the bacterial surface proteins InlA and InlB, important virulence factors for host cell invasion, have sigmaB and PrfA dependent promoters (103). Expression of these internalins increases during stationary phase without an increase in *prfA* transcription (105). *In vitro* exposure of *L. monocytogenes* to sublethal stresses such as stationary phase, osmotic stress or conditions designed to mimic the host intestine, result in an increased stress response as well as induction of *inlA*, *inlB* and a virulence associated bile salt hydrolase gene, *bsh* (103, 202).

The control of *prfA* by a sigmaB dependent promoter (promoter P2 immediately upstream of the *prfA* gene, see Figure 1.3), illustrates a direct link between sigmaB and virulence (149, 169, 187). However the loss of transcription from this promoter does not hinder invasion of host cells in tissue culture or affect virulence in oral infection of guinea pigs (67). The complete deletion of sigmaB decreases invasion of host cells in tissue culture, possibly due to the decreased levels of internalins (67, 104, 105). *In vivo*, sigmaB deletion mutants are less virulent than wildtype bacteria in the oral infection of mice and guinea pigs (67, 149). This attenuation was not seen in I.V. infected guinea pigs, leading the authors to hypothesize that sigmaB is involved in gastrointestinal infection but not important for systemic spread (67).

5. Two critical steps in virulence

The pathogenesis of *L. monocytogenes* involves two critical steps. As a food borne pathogen, *L. monocytogenes* must first overcome the intestinal barrier. The intestine provides the host with innate immunity as well as adaptive immunity. The protective immune functions of the gut are beyond the scope of this dissertation, but the role of the intestine in providing a physical barrier will be discussed. Once *L. monocytogenes* successfully invades the intestinal epithelium or any cell during infection, the bacterium must lyse the phagocytic vacuole. The phagocytic vacuole is a host defense system, and the bacterium must escape before it is killed. Key players in bacterial escape are described below.

Part II. Overcoming the intestinal barrier

1. Basic intestinal architecture

The small intestine is divided into three main sections from proximal to distal: the duodenum, the jejunum and the ileum (128, 178, 179). The intestine is organized into projections called villi, increasing the surface area by more than 10 times (Figure 1.4). The main cell type of the intestine is the absorptive enterocyte. Enterocytes are tall columnar cells that possess apical cellular modifications called microvilli that increase surface area if the intestine by an additional 20-40 times. Along the intestine are invaginations of the epithelium that form simple tubular glands called crypts. The content of crypts is sterile and crypts are responsible for sensing abnormal microbial presence. The upper half of a crypt is comprised of enterocytes and goblet cells (discussed below), while the lower half of the gland is lined in regenerative cells. These regenerative cells are the stem cells for the intestinal mucosa. As new cells are formed older cells are pushed toward the luminal surface to the tips of the villi where they are extruded. At the base of the crypts are acidophilic granule cells or Paneth cells with antimicrobial capabilities such as the secretion of cationic microbial peptides (CAMPs).

The gut also contains areas of concentrated lymphocytes known as the gut associated lymphatic tissue (GALT). In the small intestine, especially concentrated in the ileum, are structures called Peyer's Patches (PP). PP are essentially lymph nodes with flattened microfold cells or M-cells covering them. M-cells constantly sample the lumen and deliver the contents to waiting macrophages and dendritic cells in the subepithelial dome of the PP. Below these cells are T and B lymphocytes ready to induce an immune response if necessary. This sampling action is also important for

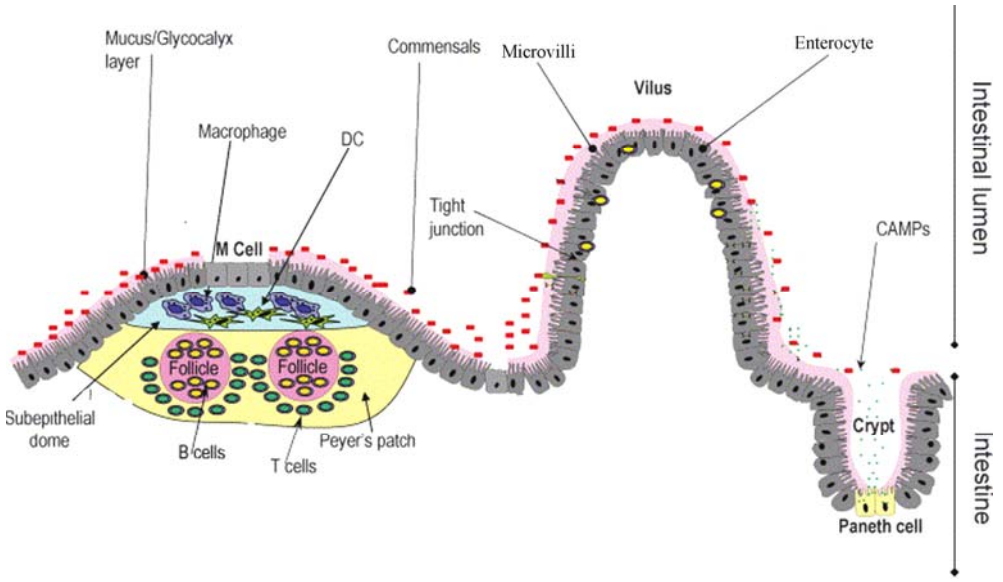

Figure 1.4. The intestinal architecture.

Adapted from Magalhaes *et al*. 2007. The intestinal epithelial barrier: how to distinguish between the microbial flora and pathogens. DC: Dendritic cell, CAMPs: Cationic antimicrobial peptides. Semin. Immunol. 19:106 – 115.

maintaining tolerance to the multitude of dietary and commensal gut flora antigens present in the intestinal tract.

2. Obstacles to pathogens presented by the intestine

The lumen of the intestine is mildly acidic and has an elevated osmolarity (37, 58). The intestinal epithelial cells form a single layer that defines the outside of the body from the inside of the body (Figure 1.4). The integrity of this barrier is due to the intimate contact between neighboring cells and is formed by tight junctions made of transmembrane proteins (Figure 1.5) (160). These tight junctions seal the apical sides of neighboring cells together. Basolaterally to these tight junctions are adherens junctions formed from homophilic calcium dependent interactions between E-cadherins. The extracellular domains of E-cadherins interact with each other while the intracellular domains bind β-catenins which in turn connect to the actin cytoskeleton. Basolaterally to the adherens junctions are desmosomes formed by interactions between desmosomal cadherins which are connected to intermediate filaments.

A continuous layer of mucus and glycocalyx is present on the apical side of the intestinal epithelium (Figure 1.4) (178). The glycocalyx is a cell attached, protective layer of polysaccharides found on the surface of the microvilli of absorptive cells. This minimizes the ability of invading microbes to reach and adhere to the epithelium. Mucus is secreted by goblet cells. These cells are scattered among the enterocytes with an increased density toward the large intestine. They are filled with glycosylated macromolecules called mucinogens that are hydrated upon secretion to become mucus. Mucus protects against microbial interaction with the intestinal epithelium. It also provides an attachment site for bacteria which become coated with mucus and can be removed by peristalsis.

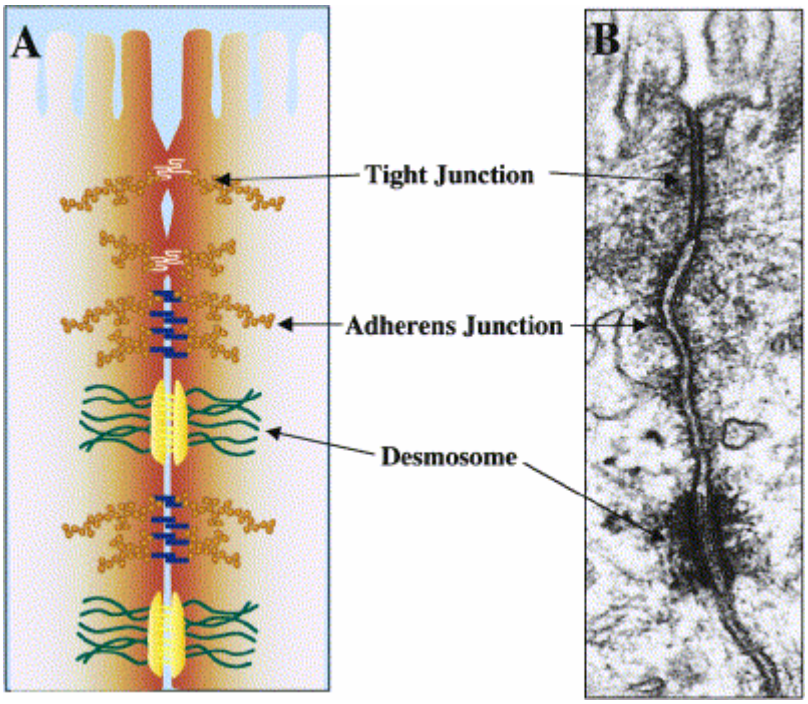

Figure 1.5. Intracellular Junctions. A) Diagram of the three major types of intercellular junctions in epithelial cells: tight junctions, adherens junctions and desmosomes. B) Electron micrograph depicting the ultrastructure of adherens junctions, desmosomes, and tight junctions between two murine intestinal epithelial cells.

From Perez-Moreno *et al*. 2003. Sticky business: orchestrating cellular signals at adherens junctions. Cell 112: 535 – 548.

The intestine is home to approximately 10^{14} bacteria comprised of around 500 species (128, 179). These bacteria are termed "commensals" and are primarily found in the lumen outside of the mucus layer. These species consist of anaerobic, aerobic and facultative aerobic bacteria; the exact composition of the community varies along the length of the intestinal tract. This relationship provides the bacteria with a stable environment and some nutrients while bacteria aid in digestion of food, modulation of the host immune system, and by blocking intrusion of pathogenic microorganisms. This is termed "colonization resistance" and involves multiple factors such as producing antimicrobials, competing with pathogens for adhesion sites, stimulating mucin secretion, and creating competition for nutrients (198).

3. Flagella structure and function

The flagellum is a complex molecular machine consisting of three major components: the basal body, the hook and a long semi-rigid helical filament (Figure 1.6) (82). Its assembly is highly regulated and hierarchical, beginning with the formation of the base, called the basal body, which anchors the flagella to the cell. Once complete the hook is added followed by the flagellin monomers that create the filament. The motor proteins which make up the force generating unit are the last components (127). Flagella can be monotrichous, present as a single flagellum at a pole, lophotrichous, meaning multiple flagella in a tuft, or peritrichous, present all around the bacterium (30). *L. monocytogenes* produces 5-6 peritrichous flagella.

Motility is achieved by rotating the flagella powered by ion motive force across the membrane. In order to successfully exploit environmental resources bacteria must move toward chemical attractants and away from chemical repellants. Chemotaxis is the system used to control bacterial flagellar rotation. Individual chemotaxis system

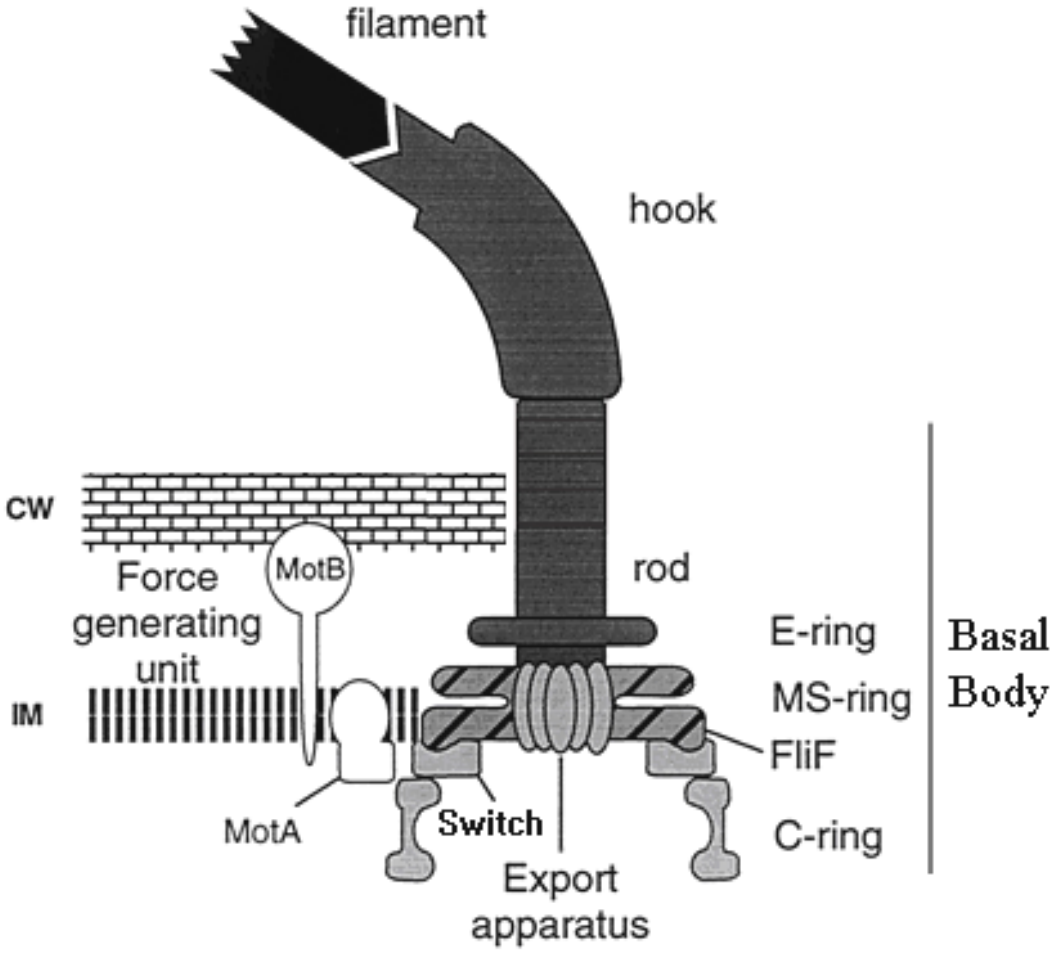

Figure 1.6. Flagellar structure in a gram positive bacterium.

Adapted from Grunenfelder *et al*. 2003. Role of the cytoplasmic C terminus of the FliF motor protein in flagellar assembly and rotation. J. Bacteriol. 185:1624 - 33.

proteins can differ between bacteria, but the general design is a two-component signal transduction system. This system generally includes transmembrane receptors for binding chemicals, an autophosphorylating sensor kinase and a response regulator. Ligand binding causes a conformational change in the receptor that is transmitted through a signal casade to the response regulator. This protein directly interacts with the flagellar switch, part of the basal body, to change the rotational direction. For peritrichous flagella clockwise rotation causes the flagella filaments to splay outward and sends the bacterium into a tumble. Counter clockwise rotation bundles the filaments together and propels the bacterium into a forward run. The probability of counter clockwise rotation is defined as bias. The chemotaxis system alters the amount of time spent in either a tumble or a run. Bacterial movement can be seen as a biased random walk causing the net migration of the bacterium along a chemical gradient. The *L. monocytogenes* chemotaxis system probably most closely resembles the system found in the gram-positive model organism *Bacillus subtilis*. For reviews see (195, 204, 215).

4. Possible roles for motility in microbial invasion

Flagella are integral to the virulence of multiple gastrointestinal pathogens (100, 154). There are three main ways flagella contribute to bacterial pathogenesis (Figure 1.7). First, flagella mediated motility can increase invasion or colonization by helping the bacterium swim toward the host cell or by enhancing the bacterial-host cell interaction. Second, the flagella filament can act as an adhesin to tether the bacterium to the host cell (71). Last, the flagellum's basal body apparatus can act as a secretion system for bacterial effectors (196, 227). These potential flagellar functions are not mutually exclusive and may be used in combination.

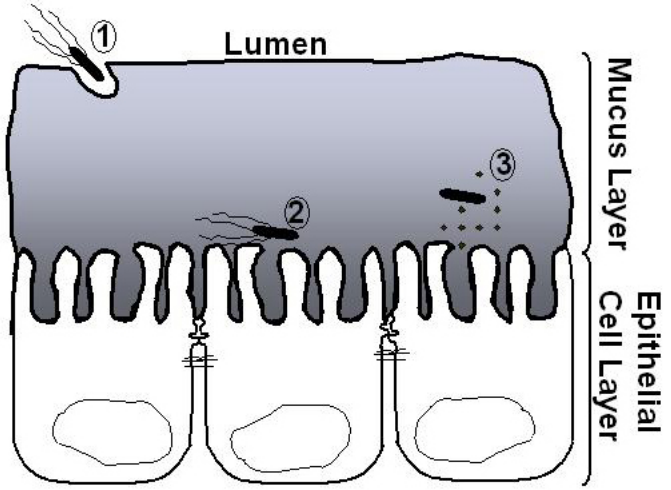

Figure 1.7. Potential functions for flagella in virulence. 1) Flagella mediated motility 2) Flagella filaments acting as adhesins 3) Secretion of bacterial effectors through the flagellar basal body

There are many examples of motility playing a role in the virulence of gastrointestinal pathogens. One well studied example is the pathogen *Helicobacter pylori*, a gram negative, spiral bacteria with 2-6 polar flagella that colonizes the stomach mucosa (13). Colonization is associated with gastric and duodenal ulcers and the development of gastric cancer (134, 157). *H. pylori* is known to reside deep within the gastric mucus away from the acidic lumen and preferentially colonizes less acidic areas of the stomach (113). While adherence to the gastric epithelium is associated with some disease outcomes, it is not required for infection and colonization (83).

Flagella-mediated motility and chemotaxis have been shown to be important for *Helicobacter* colonization of the gastrointestinal tract in many animal models (53, 59, 135, 153). There is evidence that pH serves as the chemotactic signal guiding bacteria deep into the mucosal layer, away from the acidic lumen, promoting close interaction and possible adherence between bacteria and host epithelial cells thereby eliciting inflammation (36, 135, 184).

Another pathogen that uses motility for virulence is *Vibrio cholerae. V. cholerae* is a gram negative curved rod with a single polar flagellum. Infection with *V. cholerae* causes a diarrhea described as "rice water stool", vomiting, and severe dehydration that can lead to death (177). Initial attachment of the bacteria to the host mucosa takes place in the small intestines facilitated by various outer membrane proteins, HA protease, polysaccharides and lipopolysaccharides (LPS). Production of the toxin coregulated pilus then allows for pilus-pilus interaction between bacteria and microcolony formation (108, 205). Cholera toxin, a ribosylating enterotoxin, is secreted by colonizing bacteria and is responsible for the diarrhea. The profuse

diarrhea causes the expulsion of large numbers of *V. cholerae* back into the environment (20).

The loss of motility, either by deletion of the gene for flagellin or by deletion of motor proteins generating paralyzed flagella, results in bacteria that are attenuated for colonization in an infant mouse model (119). This demonstrates that motility is advantageous for colonization. In fact, human shed *V. cholerae* are highly motile (142). Surprisingly though, comparison of transcription profiles of human shed bacteria with stationary phase *in vitro* grown bacteria shows that shed bacteria have lower levels of chemotaxis gene transcription (142). Human shed *V. cholerae* are also more infective than broth grown bacteria in an oral mouse model. These shed bacteria are less chemotactic in capillary assays and have lower levels of some chemotaxis proteins (21). It appears that motility without chemotaxis may increase infectivity.

Why would *V. cholerae* be more infectious if highly motile yet non chemotactic? It has been theorized that the temporary down regulation of chemotaxis might allow for targeted colonization of the small intestine and could reduce the infectious dose (21). It is also possible that chemotaxis is turned off in the intestine after colonization has been established to allow bacteria to stop responding to epithelial cell attractants and encourage bacterial shedding (20). Alternatively, the increase in motility may aid the bacteria after returning to the aqueous non-host environment (20). One possible way this could be regulated would be through quorum sensing. Interruption of the *V. cholera* quorum sensing response regulator gene *luxO* drastically reduces the ability of bacteria to colonize the small intestine of infant mice (228). Microarray studies of this quorum sensing mutant compared to a wildtype strain suggest that when bacterial concentrations increase and quorum sensing signals are in high concentration

virulence genes are downregulated, including the toxin coregulated pilus, and motility genes are upregulated (228). Perhaps once *V. cholera* senses that bacterial populations are robust and colonization has been well established it is advantageous to increase motility, stop producing the pilus that encourages microcolony formation, and induce shedding.

Salmonella enterica serovar Typhimurium is a gram negative motile rod with peritrichous flagella that usually causes a self-limiting gastroenteritis (151). Infection commonly results from ingestion of contaminated food or water. Invasion of host cells occurs in the small intestine. Intestinal invasion can occur through non-phagocytic epithelial cells, but bacteria preferentially invade through M-cells. Once bacteria translocate through M-cells, invasion of neighboring cells through the basolateral side can occur (85). In some inbred mice *S*. Typhimurium causes a more severe disease. This is used as a model for typhoid fever, a disease caused by *Salmonella enterica* serovar Typhi, a pathogen restricted to humans. Patients with typhoid fever have a fever, abdominal pain, diarrhea or constipation, and a rash.

A murine ligated loop model was used to investigate invasion phenotypes of chemotaxis mutants (97). Direct injection of a one to one mix of mutant to wildtype bacteria into an isolated portion of the small intestine allows for greater control over infectious dose, and using a competitive index controls for animal to animal variability. Differential counts of intracellular bacteria determined that straight swimming chemotaxis mutants are hyperinvasive, while tumbling chemotaxis mutants are attenuated. This led to the hypothesis that the physical distribution of flagella filaments around the bacterium may affect host cell invasion. Swimming forward would bundle filaments behind the bacterium promoting interactions between the

bacterial surface and the host cell. Alternatively, tumbling surrounds the bacterium with flagellar filaments, potentially interfering with host cell contact and decreasing invasion. This theory was supported by the fact that in the same model a non-flagellated mutant invaded PP only slightly less than wildtype bacteria, while flagellated but non-motile mutants were less invasive. Apparently a non-flagellated mutant is unhindered while a non-motile but flagellated mutant has paralyzed flagellar filaments impeding contact with the host cell.

While flagellar arrangement may affect host cell invasion, is motility *per se* an advantage? More recently, a streptomycin pretreated mouse model was developed. This model allows for the examination of *S.* Typhimurium induced colitis, a disease which more closely resembles the effects of *S*. Typhimurium on humans (8). Intragastric infection of streptomycin-pretreated mice with non-flagellated and wildtype bacteria results in equal bacterial counts in the cecum, mesenteric lymph nodes, spleen and liver at time points from 10 hours to 2 days post-infection. However, there is significantly less inflammation (as characterized by histological scoring of submocosal edema, polymorphonuclear leukocyte infiltration and epithelial integrity) at early time points with the non-flagellated mutant. Why would there be less inflammation with similar bacterial loads? Further analysis by fluorescence microscopy reveals that while wildtype bacteria are concentrated at the epithelial surface, non-flagellated bacteria are more abundant in the lumen. This lack of intimate contact may explain the decreased inflammation, but it is surprising that this does not translate into decreased invasion. It is possible that flagella are not required for interaction with M-cells, thereby not affecting systemic spread (197).

These three examples elucidate the multiple roles that motility can perform in gastrointestinal pathogensis. While *H. pylori's* motility helps it maintain position deep in the stomach mucosa through pH chemotaxis, it appears that non-chemotactic motility may be the most beneficial for *V. cholera* virulence. In *S.* Typhimurium motility impacts both its ability to position itself close to intestinal epithelium and to elicit inflammation but does not affect systemic spread. Clearly motility can impact virulence in a variety of ways.

5. *L. monocytogenes* factors involved in crossing the intestinal epithelium

Invasion of intestinal epithelial cells by *L. monocytogenes* is an active process that requires host cell actin rearrangement (65). Engulfment of the bacterium by a potential host cell involves bacterial ligands binding to host receptors causing cytoskeletal rearrangements in the host cell which results in phagocytosis (34). Multiple bacterial ligands have been determined and are described below. See appendix 2 for a quick reference chart of tissue culture lines.

An *in vitro* transposon mutagenesis screen for mutants deficient in invasion of human intestinal epithelial Caco-2 cells led to the first descriptions of InlA (64). InlA is an 800 amino acid protein, a member of the leucine rich repeat (LRR) family, and is anchored to the cell wall by sortase A through a LPXTG motif (11, 44). Affinity chromatography using host cell extracts and an InlA column demonstrated an interaction between InlA and the host cell protein E-cadherin (140). Host cells will phagocytose beads coated with the LRR and interrepeat region (IR) of InlA. Expression of the LRR and IR of InlA by non-invasive *L. innocua* render the bacteria invasive (117).

E-cadherin is a calcium dependent, transmembrane, cell adhesion molecule located in adherens junctions between polarized cells (Figure 1.5) (160). The extracellular domain participates in homophilic interactions with the extracellular domain of an E-cadherin on a neighboring cell. The intracellular domain is attached to the cytoskeleton through catenins. While InlA mediated adhesion of bacteria only requires the extracellular domain of E-cadherin, the cytoplasmic domain is required for invasion (116). The interaction between InlA and E-cadherin is specific and depends on the host species. The expression of chicken E-cadherin on fibroblasts not known to normally express cadherins increases invasion by *L. monocytogenes*. Antibodies to chicken E-cadherin can block this entry (140). Host cells expressing mouse E-cadherin are not permissive to *L. monocytogenes* entry (115). It was determined that this species specificity is due to the 16th residue of E-cadherin, a proline in humans, chickens and guinea pigs but a glutamic acid in rodents (115). This discovery puts into question the applicability of the traditional mouse model and a transgenic mouse expressing human E-cadherin on the surface of enterocytes has been developed (118). This transgenic mouse allows for more efficient invasion of *L. monocytogenes* across the intestine and translocation to the mesenteric lymph nodes, liver and spleen after oral infection. The guinea pig is also more susceptible to infection than the non-transgenic mouse model.

One complicating factor in the use of E-cadherin as a cellular receptor by InlA is the localization of E-cadherin in adherens junctions. These junctions are on the basolateral side of tight junctions and are presumably not accessible to bacteria in the lumen (160). In a study of a polarized epithelium in tissue culture extrusion of senescent cells was shown to occur at specialized junctions where E-cadherin is transiently exposed (159). Instead of disrupting the epithelial layer to access its

receptor, *L. monocytogenes* uses these unique sites for attachment and invasion. In a rabbit ileal loop model these sites were shown to be present at the tips of villi where cells are sloughed off as new cells are generated in crypts. *L. monocytogenes* was seen associating with these E-cadherin exposing regions.

Similar to InlA is the Virulence Protein Vip, another *L. monocytogenes* protein unique to pathogenic species that has an LPXTG cell wall anchoring motif (23). However instead of a LRR motif it has a proline rich region and is therefore not a member of the internalin family. Comparison of RNA from a wildtype versus a *prfA* deletion mutant shows higher *vip* transcripts in the wildtype strain indicating some level of positive regulation by PrfA. The Vip protein can be detected on the bacterial surface using immunofluorescence and its cellular receptor was identified as the endoplasmic reticulum chaperone Gp96. The absence of Vip decreases invasion of Caco-2 cells as well as the mouse fibroblast cell line L2071. Loss of Vip does not affect invasion of the guinea pig epithelial cell line, GPc16, or the African green monkey kidney cell line, Vero. These invasion defects correspond well with the expression of Gp96 which is found on the surface of Caco-2 and L2071 cells but not on GPc16 or Vero cells. Surprisingly, the deletion of *vip* does not affect the ability of the bacteria to adhere to L2071 cells and plaque sizes are equal to wildtype indicating no defect in intracellular growth or cell to cell spread. Unfortunately the authors did not test adherence to Caco-2 cells. It is possible that adherence to host cells is mediated by other bacterial proteins and Vip's role is to trigger host signaling events that result in enhanced bacterial entry. In the oral infection of transgenic mice a *vip* deletion mutant is less virulent as determined by bacterial counts in organs up to 72 hours.

Comparison of the *L. monocytogenes* genome with the *L. innocua* genome shows the *aut* gene to be present in the pathogen genome only (16, 22). This protein has a C-terminal GW cell wall anchoring motif, similar to the invasion protein InlB. The *aut* gene product, Auto, has an N-terminal autolysin domain. Autolysins are responsible for controlled hydrolysis of peptidoglycan during bacterial growth. Renaturing polyacrylamide gel electrophoresis confirmed autolytic activity but no defect in cell separation or antibiotic induced lysis of *aut* mutant bacteria (22). Deletion of *aut* has no effect on microscopic morphology, hemolytic activity or expression of ActA, InlA or InlB. Deletion of *prfA* has no effect on expression of *aut*, suggesting it is not PrfA regulated. Adhesion to Caco-2, Vero and Hep-2 cells is not affected by the loss of Auto. Invasion of these same cell lines, as well as guinea pig epithelial cells and a murine fibroblast line, decreases by 5-50 fold and the defect is at least as great as the effect of deleting *inlA* or *inlB*. Similar to Vip, Auto's importance for invasion but not adhesion suggests a possible role in signaling. The Auto mutant is not defective in cell to cell spread. Oral infection of guinea pigs shows the Auto mutant to have fewer bacteria in the intestine at 24 hours and less bacterial load in the intestine and liver at 72 hours compared to the wildtype.

ActA is well known for its role in host cell actin polymerization to generate bacterial cytoplasmic movement, but it also appears to have some influence on invasion of epithelial cells. The deletion of *actA* from a *L. monocytogenes* strain that overexpresses all PrfA genes (PrfA*), results in a mutant that is less invasive of epithelial cells in tissue culture (201). This decreased invasion level is still greater than wildtype without overexpression of PrfA. The contribution of ActA to invasion is only evident with high levels of ActA on the bacterial surface (147). This may have biological relevance for bacteria released from the cytosol of one host cell attempting

to invade a new host cell. Using electron microscopy it was shown that these *actA* mutants in a PrfA* background appear to elicit less actin rearrangement of Caco-2 cells than PrfA* strains expressing *actA* during invasion. Heterologous expression of ActA on the noninvasive *L. innocua* increases uptake by epithelial cells 77 times, although the absolute invasion level is still low (201).

Listeria Adhesion Protein (LAP) is found in all Listeria species except for *L. grayii* and is predominantly a cytoplasmic protein, although a small amount can be seen at the cell surface by immune-electron microscopy (94, 155). The loss of LAP decreases adhesion to the human colon cell lines Caco-2, HT-29 and HCT-8 (94, 106, 155, 218). Adhesion to a duodenum cell line, HuTu-80, as well as non-intestinal cell lines is not affected (94). Invasion of cell lines corresponds with adhesion levels. There is also decreased adhesion to Henle-407 cells, originally reported as a jejunum-ileum cell line. These different adhesion levels could suggest that LAP mediates adhesion in the lower part of the small intestine and the colon. Expression of LAP in *E. coli* increases adhesion to Caco-2 cells by 2.8 fold. LAP shows homology to an alcohol acetaldehyde dehydrogenase with an iron containing alcohol dehydrogenase domain and an aldehyde dehydrogense domain (106). The host cell receptor for LAP is Heat Shock Protein 60 and binding appears to be rapid and specific (106, 218). In an oral mouse model, LAP deficient mutants are not found in the livers of infected animals. Infection of mice by intraperitoneal injection results in no difference between the wildtype and LAP mutants, suggesting that LAPs main role may be in invasion of the intestine or possibly in survival in the gastrointestinal tract (94).

A screen for proteins important in colonization of the liver in a mouse I.V. infection model identified the *fbpA* gene (47). This transposon mutant was used in an oral

infection of a transgenic mouse model expressing human E-cadherin (118) and had lower bacterial counts than the wildtype strain in the intestine and liver. These differences were not seen in a non-transgenic mouse. Fibronectin binding protein A (FbpA), the product of *fbpA*, can be seen on the bacterial surface by immunofluorescence. Fractionation of broth grown bacteria followed by western immunoblot shows FbpA is mainly localized in the membrane fraction. A complicating factor in interpreting FbpA's role in virulence is that protein levels of secreted LLO as well as InlB are reduced in the *fbpA* mutant despite normal levels of gene transcription. It is hypothesized that FbpA has two functions, first as a binding protein and second as a chaperone for LLO and InlB. It is difficult to separate the contributions of these two potential roles.

The product of the *iap* gene is the murein hydrolase P60 (110, 111, 223). P60 expression is PrfA independent (18, 19). It contains a sec dependent signal sequence, a conserved N-terminal domain with homology to SH3 domains, a conserved C-terminal domain with homology to hydrolytic enzymes and a highly variable center domain with a series of threonine-asparagine repeats (110). It is secreted as well as associated with the bacterial surface (176).

P60 was first investigated when it was noted that spontaneous rough morphology colonies were less invasive than normal smooth colonies and expressed less P60 (91). At first the use of spontaneous rough mutants was necessary because the *iap* gene was believed to be essential (223). The spontaneous rough morphology phenotype was later attributed to the loss of the SecA paralog, SecA2 (124). Mutation in SecA2 affects the secretion of multiple other proteins making it difficult to evaluate the importance of P60 using spontaneous rough mutants.

Successful in-frame deletion of *iap* has since been accomplished (123, 161). There is still conflicting data on the effects of losing the *iap* gene though. In one study, deletion of *iap* did not affect the production of LLO, ActA, PC-PLC, PI-PLC, InlA or InlB as determined by western immunoblotting (161). It did lead to an abnormally long filamentous morphology and incomplete septation. Additionally ActA and InlA cell surface distribution was no longer polar and there was a decrease in appearance of intracellular actin tails (161). The I.V. infection of mice led to reduced recovery of the mutant in the liver and spleen at 3 days and a 30% decrease in tissue culture invasion of Caco-2 cells (161). Another study used a one to one mix of an *iap* deletion mutant to wildtype bacteria in an I.V. competitive infection of mice. This also showed an *in vivo* attenuation, as the mutant was recovered at $1/50^{th}$ the level of wildtype in livers and spleens after 48 hours. However replication in mouse bone marrow derived macrophages was normal, pseudopod formation during cell to cell spread was normal as were plaque sizes, all indicating wildtype distribution of ActA (123). It is unclear why these two groups saw different phenotypes.

L. monocytogenes pathogenesis depends in part on its ability to invade the host intestine. InlA mediated invasion of host cells through E-cadherin has been studied in great detail. However, since *L. monocytogenes* infects different animal species and cell types, it can be assumed that there are multiple bacterial ligands and a variety of host cell receptors mediating invasion. Other bacterial ligands are beginning to be investigated and more are likely to be discovered.

6. Invasion through M-cells

While *L. monocytogenes* is well equipped to cross the intestinal barrier by invading epithelial cells, intestinal invasion can also occur through M-cells. Several other

gastrointestinal pathogens, such as *Yersinia*, *Shigella* and *Salmonella*, invade through M-cells.

Oral infection of mice with *L. monocytogenes* showed an association of bacteria with PP followed by bacterial replication and dissemination to deeper organs (40, 126, 129). However, this tropism for PP has not been seen with other animal models. In a rat ligated ileal loop model invasion and translocation to organs follows the same kinetics regardless of the presence of PP in the ileal loop, although more bacterial replication is seen in PP (168). Infection of guinea pigs with *L. monocytogenes*, a system thought to more closely resemble human invasion due to the guinea pig expression of an InlA permissive E-cadherin, also does not show any preference for PP (118). Finally, in an *in vitro* model of polarized Caco-2 cells and M-cells bacteria are randomly distributed. In fact, tissue culture layers that include M-cells are overall less permissive to invasion than those tissue culture layers without M-cells (40). The constant sampling of intestinal contents by M-cells surely plays some role in *L. monocytogenes* invasion, but it is difficult to assess what contribution this makes in human infection.

Part III. Escape from the Vacuole

1. The vacuole

Upon entry into the host cell, *L. monocytogenes* are trapped in phagocytic vacuoles (65, 146, 206). As was previously described, phagocytosis of bacteria can be triggered by the binding of a variety of different receptors with bacterial ligands (156). Newly formed phagosomes are derived from the plasma membrane and are not bactericidal because they are immature. The phagosome undergoes a maturation process resulting

in a phagolysosome that can kill and degrade trapped bacteria (52, 84, 199, 213). This phagolysosome biogenesis is an extremely complex process that involves multiple fusion events with other vesicles that alter the membrane protein and lipid composition as well as the lumen contents (42, 43). The maturation of the phagosome depends on interactions with the endocytic pathway (Figure 1.8). In brief, soon after the phagosome forms it acquires markers of early endosomes, such as the small GTPase molecular switch Rab5 and the trafficking protein Early Endosomal Antigen-1, from the fusion with early endosomes. As fusion and fission events continue Rab5 is lost and later endosomal markers are acquired such as Rab7 and lysosome-associated membrane proteins (LAMPs) while at the same time the overall lipid composition changes. During the maturation process vacuolar ATPases are acquired. These pumps use the hydrolysis of ATP to move protons across the membrane and decrease the lumen pH of the compartment to a pH of less than 5. The mature phagosome also harbors multiple hydrolases to degrade and digest the phagocytosed material. Activated macrophages have an enhanced ability to kill phagocytosed bacteria in part due to the presence of NADPH oxidase complexes and nitric oxide synthases on the phagolysosomal membrane. These complexes generate reactive oxygen radicals all of which damage the engulfed pathogen. In order for *L. monocytogenes* to proliferate and cause disease it must escape from the phagocytic vacuole into the host cytoplasm. Escape from the vacuole involves multiple bacterial factors, the best described are discussed below.

2. Listeriolysin O

Listeriolysin O (LLO), the product of the *hly* gene, is not found in non-pathogenic *Listeria* species and was the first virulence factor described in *L. monocytogenes* (66, 141). It is a pore-forming toxin and a member of the cholesterol-dependent cytolysins

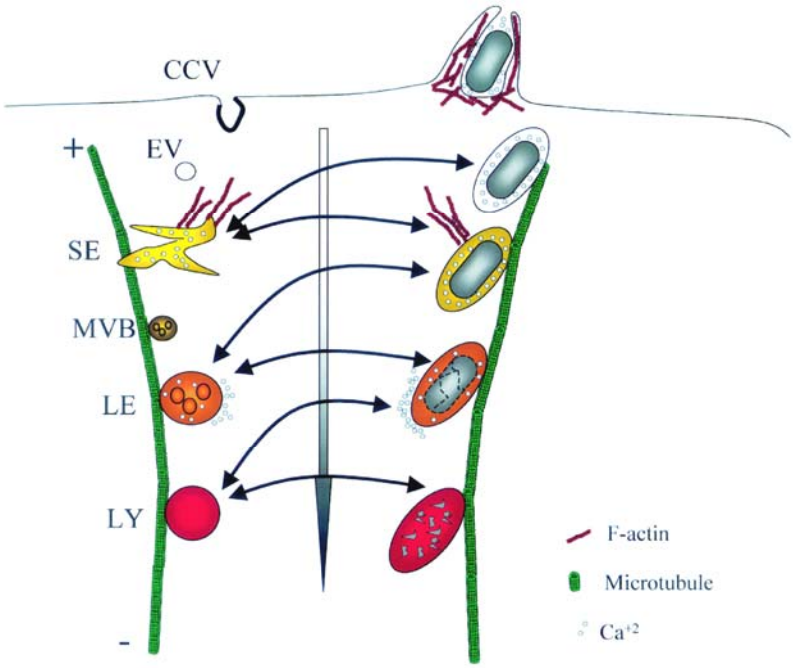

Figure 1.8. Phagosome to phagolysosome maturation parallels the endocytic pathway. The fusion of phagosomes (on right) with compartments of the endosomal pathway (on left) is indicated by the connecting arrows. CCV, clathrin-coated vesicle; EV, endocytic vesicle; SE, sorting endosome; MVB, multivesicular body; LE, late endosome; LY, lysosome.

Adapted from Vieira, O. V. *et al.* 2002. Phagosome maturation: aging gracefully. Biochem. J. 366:689 - 704.

family (CDCs) and is homologous to streptolysin O, perfringolysin O (PFO) and pneumolysin (69, 137). However, LLO is unique in its activity on host cells from the cytoplasm as other CDCs are secreted by extracellular pathogens and act on host cells from the outside (164).

Transposon mutants in *hly* are severely attenuated *in vivo* as LLO is required for escape from the phagocytic vacuole (65, 66, 146, 206). Expression of LLO by *B. subtilis* renders the bacteria able to escape from phagocytic vacuoles and grow in the host cytoplasm (10). While hemolysin mutants are unable to replicate successfully in most cell types, there are several exceptions in human cell lines (89, 152, 158, 165).

Since LLO is required for escape from the primary vacuole in most cell lines, it has been difficult to assess its potential roles in later steps of virulence such as escape from the secondary vacuole for cell to cell spread. The coating of purified LLO on the outside of *hly* mutant bacteria provides the bacteria with enough hemolytic activity to mediate escape from the primary vacuole (68). These bacteria replicate normally in the cytoplasm, form actin tails and are able to make pseudopods. These bacteria are not able to escape the secondary vacuole though, confirming the importance of LLO in cell to cell spread. Another approach to study the contribution of LLO to spreading is to place LLO under an inducible promoter in a *hly* minus background (39). LLO production can be turned on to allow escape from the primary vacuole only. These bacteria replicate and have actin tails but form small plaques as they are unsuccessful in cell to cell spread

An interesting aspect to LLO activity is the importance of the activity being compartmentalized. LLO is pH sensitive; this has been show to be an important way

to regulate activity (207). LLO is most active at acidic pH, similar to what would be found in the vacuole but not the cytoplasm, and denatures at neutral pH (69, 107, 186). The mutation of leucine 461 to a threonine results in an LLO molecule that is 10 times more active at neutral pH due to increased stability (73, 186). This mutant is still able to escape from the vacuole, but during cytoplasmic growth the host membrane becomes permeablized and the host cell is killed. This translates into decreased virulence in a mouse model (72, 73). A similar phenomenon is seen when LLO is replaced with PFO which is active at neutral pH (98, 219). PFO expressing *L. monocytogenes* are able to escape the phagosome and replicate, but the host cell membrane is compromised during cytosolic growth. PFO mutants with a more acidic pH optimum do not show this toxic effect on host cells (99).

The presence of an N-terminal PEST-like sequence is another way to compartmentalize LLO activity (41, 125). PEST sequences are regions of proteins that are rich in proline, glutamic acid, serine and threonine. These proteins have short intracellular half lives (175). Deletion of the LLO PEST sequence does not affect LLO activity, bacterial invasion, or the ability of bacteria to escape from the primary phagocytic vacuole. However, this mutant LLO does compromise the host cell membrane during cytoplasmic growth and results in host cell lysis. The LD_{50} in a mouse model indicates the mutant to be four orders of magnitude less virulent.

PEST sequences are normally used to target eukaryotic proteins for degradation. Phosphorylation of PEST sequence proteins is often a prerequisite to degradation. LLO's PEST sequence contains three putative sites for phosphorylation (41, 183). Mutation of these sites does not affect *in vitro* hemolytic activity, but these bacteria are toxic to host cells and less virulent in mice, suggesting that these phosphorylation

sites are important. However, these PEST mutant LLO proteins have intracellular half-lives equal to wildtype LLO, suggesting that the PEST motif of LLO does not control its degradation by the host proteasome (183). Cytotoxic LLO PEST mutants have equal transcript levels as wildtype LLO, but increased intracellular protein levels (182). Apparently the PEST motif negatively regulates LLO translation; the exact mechanism is still unknown.

The main role of LLO appears to be in mediating escape from the phagocytic vacuole. However, LLO may have a role in invasion of host cells. While no defect in invasion of most tissue culture cells is seen in *hly* minus mutants; there is a small decrease in invasion of the hepatic cell line Hep-2 (48, 112, 165). The addition of *L. monocytogenes* to macrophage-like J774 cells causes spikes in host cell cytosolic calcium levels (216). LLO plays a role in this pre-invasion communication between the bacterium and the host cell as an LLO mutant does not elicit these calcium changes. LLO mutant bacteria are actually internalized more rapidly than wildtype bacteria by J774 cells but are subsequently unable to escape the phagosome. Additionally, the deletion of *hly* in a background that overexpresses virulence genes, PrfA*, brings invasion levels down to a wildtype level from the elevated PrfA* level (147). It was hypothesized that LLO may play a role in invasion when cytosolic bacteria already expressing high levels of virulence genes are released from one host cell and attempt to invade a new host cell (147).

3. Phospholipases

L. monocytogenes phospholipase activity was first attributed to LLO but was eventually determined to be distinct from the hemolytic activity (95). *L. monocytogenes* produces two phospholipases, PI-PLC and PC-PLC. Both cleave

phospholipids at the glycerol to phosphorous bond, resulting in release of a phospho-head group and a diacylglycerol, designating them as members of the phospholipase C group (181).

PI-PLC, encoded by *plcA*, is a phosphitidylinositol-specific PLC with sequence homology to the PI-PLC of *B. thuringensis* (25, 75, 136). The addition of *plcA* to *B. subtilis* generates PI-PLC secretion and activity (25). The *plcA* gene for PI-PLC was first pulled out of a transposon mutagenesis screen for mutants deficient in cell to cell spread (203). PI-PLC was then described as a major virulence factor by several groups, however it was later determined that these phenotypes were actually due to a polar effect of the mutations on the *prfA* gene which is directly downstream (25, 120, 136). The two genes can be transcribed independently or as a bicistronic message (Figure 1.3). Once the in-frame deletion of the PI-PLC gene, *plcA*, was constructed the true effect of PI-PLC on virulence could be assessed (26).

The in-frame deletion of *plcA* indicates a more minor role for PI-PLC in virulence than the original studies. Cell to cell spread, as measured by plaque size, is affected only slightly by the loss of PI-PLC (26, 193). *In vivo* measurements of virulence by determining LD_{50} for I.V. infected mice also indictes a small attenuation (26, 193). The competitive infection of a mouse model shows equal amounts of wildtype and *plcA* mutant bacteria in the spleen but significantly more wildtype bacteria in the liver at 48 hours (26). Microscopic evaluation of infected mouse bone marrow derived macrophages show the mutant has a slight growth defect and is trapped in the primary phagocytic vacuole more often than wildtype (193). A PI-PLC catalytically dead point mutant has a similar characteristics to the *plcA* deletion mutant, indicating that PI-PLC activity is necessary for the wildtype phenotype (7). Host intracellular

calcium signaling is affected by the deletion of *plcA* as well. Wildtype infection elicits multiple spikes in cytosolic free calcium, but the mutant only shows a single, delayed, small increase (216). Vacuolar escape and intracellular growth from the human epithelial Henle 407 cell line is unaffected by the loss of PI-PLC (130, 193).

PC-PLC is encoded by the *plcB* gene and is homolgous to the zinc-dependent phosphatidyl choline PLC of *B. cereus* (209). The *plcB* gene is transcribed under the control of the *actA* promoter and consequently is PrfA regulated (60, 102, 138, 191). It is functional in a pH range from 5.5 to 8.0 and has activity on a broad spectrum of phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and sphingomyelin (70, 74). It is synthesized as an inactive preproenzyme of 289 residues (Figure 1.9) (150, 209). It contains a Sec-dependent signal sequence that is cleaved off, secreting a 264 residue inactive proenzyme across the bacterial membrane (131). Activation of this proezyme requires the cleavage of the 24 residue propeptide and is associated with the metalloprotease, Mpl, discussed below. Deletion of the propeptide still results in active enzyme, suggesting that the propeptide is not required for proper folding (225).

Interruption of *plcB* decreases plaque size and results in bacteria accumulating in secondary vacuoles (193, 209). Mice infected with a PC-PLC mutant have considerably lower bacterial loads in livers and spleens (26, 170). PC-PLC clearly plays an important role in escape from the double membrane vacuole during cell to cell spread. This contribution is especially pronounced in cell lines where LLO does not mediate escape from the vacuole such as Henle 407 (165). The growth rate of LLO minus mutants in Henle 407 cells is similar to the wildtype rate and immunofluorescence microscopy can be used to determine what proportion of bacteria

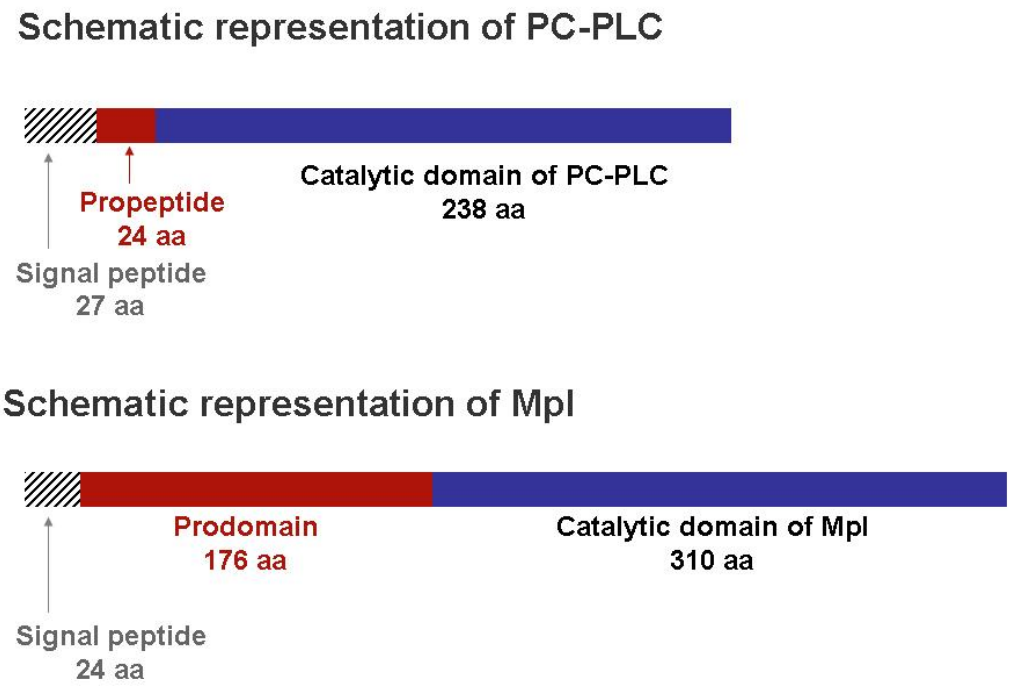

Figure 1.9. Schematic representation of PC-PLC and Mpl.

are cytoplasmic and what proportion remain in the vacuole. The loss of LLO is accompanied by a twofold decrease in the ability to escape from the primary invasion vacuole while the additional loss of PC-PLC in these mutants renders them entirely unable to escape (130). An inducible PC-PLC strain can be used to evaluate the role of PC-PLC without control by the PrfA promoter (81). Continuous, high levels of PC-PLC are necessary to mediate escape of LLO minus bacteria in cell lines that do not require LLO (81).

These two phospholipases have overlapping functions. The deletion of PI-PLC results in a 10% decrease in plaque size and a two fold increase in mouse LD_{50}. The deletion of PC-PLC shows a 34% decrease in plaque size and a 20 fold increase in mouse LD_{50}. The deletion of both phospholipases in the same strain has a much larger effect. Plaque size decreases 68% while the mouse LD_{50} increases 500 fold (193). The actions of these two phospholipases appear to partially complement each other and are both necessary for full virulence.

The regulation and compartmentalization of PC-PLC activity is critical to virulence. The proenzyme of PC-PLC (ProPC-PLC) is stored at the bacterial cell wall / membrane interface (Figure 1.10) (132, 194). The activation of proPC-PLC is mediated by Mpl (167, 170). However there is a host protease that is able to activate proPC-PLC during cell to cell spread (131). Bacteria maintain a pool of proPC-PLC that is activated and released as a bolus (132). This activation and translocation across the cell wall takes place in acidified vacuoles (131, 132). The translocation is dependent on the presence but not on the cleavage of the propeptide as mutation of the cleavage site results in a proform that is still translocated in a Mpl and pH dependent manner (225). The deletion of the PC-PLC propeptide results in an active PC-PLC

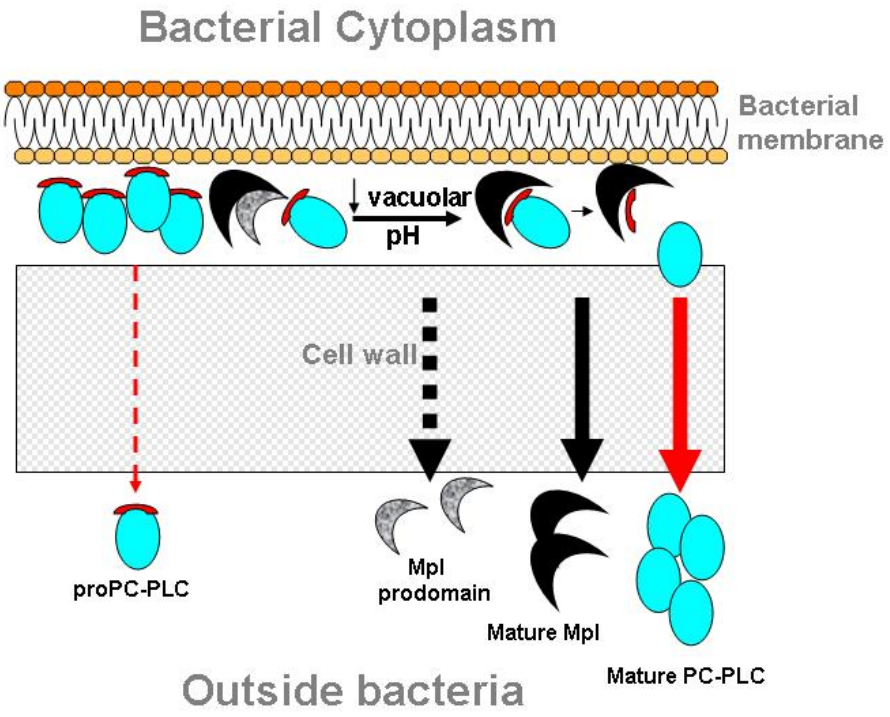

Figure 1.10. Model for Mpl mediated processing and translocation of PC-PLC across the bacterial cell wall. Pool of proform PC-PLC is maintained at bacterial cell wall membrane interface and is released as a bolus of mature PC-PLC upon a decrease in pH. Mpl mediates the processing and the translocation of PC-PLC. Thickness of arrow depicts increasing translocation of protein.

that is constitutively secreted, regardless of pH and Mpl (225). This constant secretion of active PC-PLC compromises host cell membranes during intracellular growth, specifically at the site of spreading filopodia (224). Loss of this compartmentalization of activity to the vacuole renders the mutant 200 fold less virulent in a competitive infection of mice (224).

4. Metalloprotease

The *L. monocytogenes* metalloprotease, Mpl, was discovered during a transposon screen for phospholipase negative mutants (139). Mpl has a HEXXH motif and is a member of the M4 family of zinc metalloproteases represented by thermolysin from *B. thermoproteolyticus* (145). Thermolysin is composed of two domains that come together to form a cleft containing the active site with a zinc ion and four calcium binding sites (92).

Mpl is PrfA regulated and part of the *Listeria* pathogenicity island-1 (Figure 1.3) (101, 138). Similar to PC-PLC it is also translated as a preproenzyme with a Sec dependent signal sequence, a large 176 amino acid propeptide and a 310 amino acid protease domain (Figure 1.9) (12, 139). Maturation of the inactive proprotein occurs exclusively by intramolecular autocatalysis (12). The Mpl protease is active over a pH range of 5.0 – 9.0 with an optimum at 7.0 (31).

Mpl has multiple functions. First, as described above, Mpl is associated with the proteolytic activation of the PC-PLC proenzyme. Interruption of Mpl eliminates production of active PC-PLC (139, 150, 167, 170). Mpl activity is required for this maturation function as a catalytically dead Mpl is unable to activate PC-PLC (12). Second, it controls the translocation of PC-PLC across the cell wall and loss of Mpl

stops PC-PLC translocation (225). Again, a catalytically inactive mutant is unable to perform this translocation function (12). These two functions, activation and translocation of PC-PLC, are related but are in fact independent of one another. The activation of PC-PLC is not required for PC-PLC translocation by Mpl. A PC-PLC mutant that cannot be cleaved by Mpl is still translocated in a manner dependent upon Mpl and upon pH (225). Mpl's ability to regulate this translocation is dependent on the presence of the PC-PLC propeptide. Deletion of the PC-PLC propeptide results in translocation independent of Mpl and pH (225).

The third function of Mpl is the degradation of ActA. This degradation is pH dependent (172). The hypothesis is that degradation of ActA by Mpl would happen in the secondary spreading vacuole, and probably allows for bacterial replication to occur before actin polymerization and bacterial movement start (172).

Mpl's role in virulence is related to its ability to activate and translocate PC-PLC. Invasion and intracellular growth of Caco-2 and J774 tissue culture cells is not affected by the loss of Mpl (170). Plaques are 71% the size of wildtype plaques, indicating a role in cell to cell spread (131). Mpl mutants used in I.V. infection of mice are more easily cleared from the liver and spleen than wildtype bacteria (170). LD_{50} of I.V. infected mice was only slightly increased for the mutant in one study, and not at all for an *mpl* deletion mutant in a similar study (131).

5. Model of escape

Efficient escape from the primary vacuole requires active LLO and PI-PLC (65, 193). After engulfment takes place the vacuole is acidified to a pH of about 5.9 and undergoes accelerated acquisition of early endosome markers and shows delayed

fusion with lysosomes (2, 9). The acidification of the phagosome is accompanied by perforation of the membrane by LLO (9). Preventing acidification by inhibiting vacuolar proton ATPases with bafilomycin A completely stops membrane permeabilization (9, 33). The prevention of phagosomal maturation is an active process and does not occur with dead *L. monocytogenes* and is dependent on LLO (2, 88).

Pore forming toxins such as LLO bind membranes as monomers then oligomerize to form large pores. It is unlikely though that the primary action of LLO in vacuolar escape is simply through physical disruption of the membrane (164). The amount of LLO that would be required is prohibitive, and once perforation begins the vacuole neutralizes and LLO activity would decrease (9). The ability to halt acidification of the vacuole may contribute to stopping phagosome maturation. It has been suggested that LLO created pores in the vacuolar membrane, alters intravacuolar pH and calcium levels, slowing the fusion of the vacuole with lysosomes, and allowing time for bacterial escape (88). LLO pores may also provide a channel for the passage of proteins and/or small molecules (76, 164). PI-PLC is also required for full permeabilization of phagosomes, and PI-PLC mutants are deficient in permeabilization and escape (166). In one proposed model PI-PLC would translocate through pores created by LLO to dissolve the phagosome membrane from the opposite side as well as induce host cell signaling (192). Together these two virulence factors affect host functions such as changes in calcium levels and activation of host phospholipases (77, 166, 216, 217).

The secondary spreading vacuole presents a different challenge for *L. monocytogenes*. The bacteria are now required to permeabilize a double membrane vacuole. LLO, PI-

PLC and PC-PLC all play an important role in this escape (39, 68, 193, 209). In the human cell lines where LLO is not required, PC-PLC seems to compensate as was previously discussed. A recent study of mouse bone marrow derived macrophages using an inducible LLO attempted to elucidate what role each bacterial factor played (1). Without LLO bacteria are able to spread into neighboring cells but are unable to escape secondary vacuoles, however they are mostly trapped by only one membrane. The loss of PC-PLC or both PLCs also decreases escape from secondary vacuoles, but most of the mutant bacteria are trapped in double membrane vacuoles. This led to the hypothesis that LLO is important for dissolution of the outer membrane in a double-walled secondary vacuole and that the PLCs are important for dissolution of the inner membrane.

IV. Brief Outline of Dissertation Research

L. monocytogenes is a food borne, intracellular pathogen. Successful infection therefore requires completion of two critical steps: penetration of the intestinal epithelium and escape from the phagocytic vacuole.

Motility is a well known virulence factor for many gastrointestinal pathogens but has not been extensively studied in *L. monocytogenes*. The study presented in **Chapter 2** of this dissertation explores the role of motility in host cell invasion. A previously published study suggested that flagella were actually used as adhesins (46). However, we observed that flagellated but non-motile bacteria are not as invasive as motile bacteria, indicating the importance of motility itself. We were also able to show a role for motility in early invasion of the intestine in an oral mouse model. **Chapter 3** examines the role of the metalloprotease propeptide in compartmentalization of the

protein. Compartmentalization of activity has been shown to be important for both LLO and PC-PLC. We show that loss of the propeptide results in Mpl's aberrant secretion from the bacteria and its inability to function intracellularly despite the fact that the enzyme is functional *in vitro*. **Chapter 4** summarizes our findings and discusses potential future work.

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CHAPTER 2

LISTERIA MONOCYTOGENES FLAGELLA ARE USED FOR MOTILITY, NOT AS ADHESINS, TO INCREASE HOST CELL INVASION.*

* Reprinted from Heather S. O'Neil and Hélène Marquis. *Listeria monocytogenes* Flagella Are Used for Motility, Not as Adhesins, To Increase Host Cell Invasion. Infect. Immun. 74:6675-6681.

ABSTRACT

Flagellar structures contribute to the virulence of multiple gastrointestinal pathogens either as the effectors of motility, as adhesins, or as a secretion apparatus for virulence factors. *Listeria monocytogenes* is a foodborne, gram-positive pathogen that uses flagella to increase the efficiency of epithelial cell invasion (2, 6). In this study, we aimed to elucidate the mechanism by which flagella contribute to *L. monocytogenes* invasion. To examine the role of flagella as adhesins, invasion and adhesion assays were performed with flagellated motile and non-motile bacteria, and non-flagellated bacteria. We observed that flagellated but non-motile bacteria do not adhere to or invade human epithelial cells more efficiently than non-flagellated bacteria. These results indicated that flagella do not function as adhesins to enhance the adhesion of *L. monocytogenes* to targeted host cells. Instead, it appears that motility is important for tissue culture invasion. Furthermore, we tested whether motility contributes to early colonization of the gastrointestinal tract using a competitive index assay in which mice were infected orally with motile and non-motile bacteria in a one to one ratio. Differential bacterial counts demonstrated that motile bacteria out compete non-motile bacteria in the colonization of the intestines at early time points post-infection. This difference is also reflected in invasion of the liver 12 hours later, suggesting that flagella-mediated motility enhances *L. monocytogenes* infectivity soon after bacterial ingestion *in vivo*.

INTRODUCTION

Listeria monocytogenes is a saprophytic, gram-positive rod, ubiquitously distributed in the environment. It is the etiologic agent of listeriosis, a food-borne disease affecting humans and a variety of vertebrates (26). Listeriosis occurs primarily in immunocompromised individuals causing septicemia, meningitis and

meningoencephalitis, and in pregnant women causing spontaneous abortion. Healthy adults may suffer a febrile gastroenteritis from ingesting large numbers of *L. monocytogenes* (5). The bacterium's unique ability to withstand and even thrive under a variety of stress conditions generally considered to be food preservatives makes *L. monocytogenes* of particular concern to food producers, regulatory agencies, handlers and consumers (20).

Flagella and flagella-mediated motility are integral to the virulence of multiple gastrointestinal pathogens (10, 16). There are three main ways the flagella can contribute to bacterial pathogenesis. The flagellum can serve as a secretion apparatus for virulence factors, similar to a type III secretion system. For example, *Yersinia enterolitica* secretes a virulence-associated phospholipase A, YlpA, through the flagellar apparatus (28). Flagella can also serve as adhesins to tether bacteria to host cells much like fimbrial adhesins. For instance, the flagella of enteropathogenic *Escherichia coli* contribute to bacterial adhesion to epithelial cells in a manner that is independent of motility (7). More intuitively, flagella can function as a motility and chemotactic device targeting bacteria to specific areas of the gastrointestinal tract as demonstrated for *Helicobacter pylori*. Colonization of the stomach by *H. pylori* is dependent on spatial orientation of motile bacteria within the pH gradient of the gastric mucus (21). Bacteria do not adhere to the stomach epithelium, instead localizing close to it to avoid being washed away in the lumen. Overall, the roles of flagella in bacterial pathogenesis are diverse, although not necessarily mutually exclusive.

L. monocytogenes produces 5-6 peritrichous flagella. Motility genes are down regulated at 37°C *in vitro*, although there is variation from strain to strain (8, 17, 27).

Temperature dependent regulation of motility and chemotaxis genes can be partially attributed to the negative regulator of motility gene expression, MogR (8). A *mogR* deletion mutant is strongly attenuated in mice infected intravenously, though non-motile mutants are not (27), suggesting that a mutation in *mogR* affects more than motility.

Recently, it was reported that flagella contribute to *L. monocytogenes* adhesion and invasion of human intestinal epithelial Caco-2 cells. Non-motile mutants lacking either the entire flagellar apparatus (Δ*fliF* and Δ*fliI*) or only the flagellar filament (Δ*flaA*), and a chemotactic mutant (Δ*cheY/A*) that constantly tumbles were many fold less adherent and invasive than the parent strains (2, 6). These results could suggest that the flagellum is used as an adhesin. Alternatively, motility may contribute to cell adhesion and invasion by increasing the probability of bacteria-host cell contact or perhaps by influencing the spatial orientation of bacterial interaction with host cell membranes. In fact, centrifugation of the Δ*flaA* and Δ*cheY/A* mutants onto host cells only partially complemented their adhesion and invasion defect (6). It would be advantageous for bacteria to interact head on with host cell membranes as internalin A, a major cell surface adhesin of *L. monocytogenes*, accumulates at the bacterial poles (19). In addition, the physical force generated by unidirectional movement may enhance host cell uptake as was shown for intercellular spread of bacteria that use an actin-based mechanism of motility (15).

An *in vivo* role for flagella in the pathogenesis of *L. monocytogenes* has been elusive. The non-flagellated and chemotaxis mutants described above behave similarly to their parent strains when given orally to mice as determined by bacterial counts from spleens, mesenteric lymph nodes (MLN), or intestinal mucosa at one and three days

post-infection, or from livers at three days post-infection (2, 6, 27). However, one group reported a significant difference at day three post-infection, with the Δ*flaA* mutant being recovered in higher numbers than the wild-type strain from the spleens of infected mice (6). This difference was not observed at day one or day seven post-infection. Overall, these results suggest that flagella-mediated motility is not important for systemic listeriosis in mice infected *per os.*

In this study, we aimed to elucidate the mechanism by which flagella contribute to *L. monocytogenes* invasion of human epithelial cells, and the influence of bacterial motility on early colonization of the intestinal tract in mice. Adhesion and invasion assays were performed with flagellated motile and non-motile bacteria and non-flagellated bacteria. Our results clearly indicate that flagella function as a motility device, but not as adhesins, to enhance *L. monocytogenes* invasion of epithelial cells. Moreover, we show that non-motile bacteria do not compete well with motile bacteria for initial colonization of the intestinal tract and early invasion of the liver after oral infection of mice with *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. monocytogenes* strains used for this study are listed in Table 2.1 and were routinely cultured in brain heart infusion broth (BHI) with or without streptomycin (200 μg/ml). *E.coli* DH5-α carrying pKSV7 (23) was cultured in Luria Bertani broth (LB) plus ampicillin (100 μg/ml), whereas *E.coli* SM10 carrying pPL2 (12) was cultured in LB plus chloramphenicol (25 μg/ml). *L. monocytogenes* strains carrying pKSV7 or pPL2 were cultured in BHI plus

chloramphenicol (10 μg /ml). In preparation for tissue culture assays and flagella staining, *L. monocytogenes* was cultured overnight in BHI broth, 250rpm, 30ºC.

Construction of in-frame deletion mutants. In-frame deletion mutants were generated by site-directed mutagenesis with overlap extension (SOEing) in the 10403S wild-type background strain (Table 2.1) (9). Two sets of primers were designed to amplify DNA from regions upstream and downstream of the segment to be deleted and to incorporate restriction sites (Table 2.2). These two PCR products were used in a SOEing PCR with the outside primers to create a product containing the deletion. This product was digested with BamHI and KpnI, and ligated into the shuttle vector pKSV7 and transformed into DH5-α. Purified plasmids were used to sequence cloned fragments to ensure no additional mutations had occurred. Each construct was electroporated into 10403S to replace the wild type allele with the deletion by allelic exchange (4). Deletions within the appropriate genes were confirmed by analysis of fragment sizes generated by PCR.

Construction of the MotB point mutant, *Lm motB*D23A. The *motB* gene was mutated by SOEing PCR, substituting the aspartic acid at position 23 for an alanine. Amplification of the upstream segment was performed from 10403S genomic DNA with the primer pair Marq278 and Marq283 (Table 2.2). Marq278 incorporates a BamHI restriction site and Marq283 provides the necessary codon change as well as a silent mutation to create a novel HincII restriction site for screening. Amplification of the downstream segment was performed from 10403S genomic DNA with the primer pair Marq282 and Marq 279. Marq282 also includes the necessary codon change and the HincII site. Marq279 incorporates a KpnI restriction site. These two amplification products were then used in a SOEing PCR reaction with Marq278 and Marq279. This

Table 2.1 *L. monocytogenes* strains

Strain	Genotype	Description of Mutation	Reference
10403S	Wild type (serotype 1/2a)	None	(3)
DP-L3903	10403S *erm*$^{+}$	10403S tn*917*	(1)
HEL-304	Δ*flaA*[a]	840-bp in-frame *flaA* deletion	This study
HEL-447	Δ*flaA* *flaA*$^{+}$	Hel-304 plus *flaA* ORF integrated into tRNA^{arg} site	This study
HEL-487	Δ*flaA erm*$^{+}$	Hel-304 transduced with Tn*917* from DP-L3903	This study
HEL-742	*motB*D23A	Substitution of aspartic acid 23 for alanine	This study
HEL-758	*motB*$^{+}$	HEL-742 complemented with the wt *motB* allele	This study

[a] All mutants are derived from wild-type strain 10403S

Table 2.2 PCR primers

Gene	Primer	Sequence 5'→ 3'
ΔflaA	Marq114	CGGGATCCGTGAAGAGAAGACGATTTTTATTG
	Marq115	TGAATTTGATATGTTATTAGCTGTTAGTATTTACTTTCATTTGTGTTTCC
	Marq116	GGAAACACAAATGAAAGTAAATACTAACAGCTAATAACATATCAAATTCA
	Marq117	GCGGTACCGAACGGAAAATTTCACTTACTAAC
*motB*D23A	Marq278	CGGGATCCAGTTGTAGATGGTCAATCTTC
	Marq279	GCGGTACCCACTTAGTTCCCAGTTGGAA
	Marq282	TTCATATAGTGCTTTGTTGACACTT
	Marq283	AAGTGTCAACAAAGCACTATATGGA
flaA+	Marq169	CGGGATCCGATATAAAGCCGATATTTCG
	Marq170	CGTGGATCCCTTAACATTGGCTCTGTGC

product was digested with BamHI and KpnI, and ligated into the shuttle vector pKSV7 and transformed into DH5-α. Purified plasmids were used to sequence the cloned fragment to ensure no additional mutations had occurred. The construct was electroporated into 10403S to replace the wild type *motB* allele with *motB*D23A by allelic exchange (4). Mutants were initially screened for loss of motility on motility agar plates. The *motB* gene from non-motile mutants was amplified by PCR and digested with HincII to confirm the allelic exchange.

Complementation of *Lm* Δ*flaA* mutant. The *flaA* deletion strain was complemented using the site-specific shuttle integration vector pPL2 (12). The *flaA* ORF and promoter region were PCR amplified using forward primer Marq169 reverse primer Marq170. This PCR product was digested with BamHI and KpnI, ligated into pPL2 and transformed into *E.coli* SM10 creating HEL-449. This plasmid construct was transferred into *L. monocytogenes* strain HEL-304 by conjugation. In short, log phase cultures of HEL-304 and HEL-449 were washed, concentrated ≈10 fold, mixed in a ratio of 1:2 in a final volume of 40 µl LB, and spotted onto BHI agar plates with oxacillin (8 µg/ml) (25). After a 5-hour incubation period at 30ºC, plates were washed with 1ml of LB and 100 µl was plated onto BHI agar with streptomycin (200 µg/ml) and chloramphenicol (20 µg/ml). Colonies were screened for gain of motility on motility agar plates. Motile colonies were screened by PCR using primers NC16 and PL95 (12) to amplify a 499-bp product, confirming integration of the vector.

Complementation of *Lm motB*D23A mutant. The *Lm motB*D23A mutant strain was complemented by inserting a wild-type copy of the *motB* gene back into the chromosome by allelic exchange. The wild-type copy of the gene was amplified using forward primer Marq278 and reverse primer Marq279. The PCR product was digested

with BamHI and KpnI and ligated into the allelic exchange vector pKSV7 and transformed into DH5-α. Purified plasmid was used to sequence the cloned fragment to ensure no additional mutations had occurred. The construct was electroporated into Hel-742 for allelic exchange. Successful allelic exchange was confirmed by screening for wild-type motility on motility agar and chloramphenicol sensitivity.

Flagella staining. Bacteria were stained with crystal violet (11) and viewed by bright-field microscopy with a 60X oil immersion lens. Digital photographs were taken with an Olympus DP70 digital camera.

Western blotting. Bacterial cell surface proteins were extracted by boiling the equivalent of 10ml of washed bacteria in 200 µl of 2X sample buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol) for 5 min. Extracted proteins were resolved on 12% SDS-polyacrylamide gels, and transferred to PVDF using a semi-dry electroblotting apparatus. The protein blot was reacted with rabbit immune serum to *L. monocytogenes* flagella (H-AB from Denka Seiken Co, Ltd.) at a 1/100 dilution followed by a goat anti-rabbit IgG conjugated to alkaline phosphatase (24 ng/ml) (Jackson ImmunoResearch Laboratories, Inc). Enzymatic reactivity was detected with nitroblue tetrazolium (0.33 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.17 mg/ml).

Motility agar. An LB/0.4% agar plate was stab inoculated from isolated colonies and incubated at room temperature for 36 hours. Motility was assessed by the radius of the growth ring.

Adhesion and invasion assays. Caco-2 cells were maintained in Eagle's Minimal Essential Medium (EMEM) plus 20% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. For some invasion assays, thirty-five mm tissue culture dishes were seeded with $3x10^5$ Caco-2 cells in antibiotic-free medium, but for most assays twenty four-wells tissue culture plates were seeded with $5x10^4$ Caco-2 cells per well in antibiotic-free medium. Cells were seeded on 12 mm diameter glass coverslip treated with type I rat tail collagen (0.1 mg/ml). Cells were incubated for 24 hours at 37ºC, 5% CO_2 allowing a semi-confluent monolayer to form. Cells were infected with a MOI of approximately 500, and, when indicated, one plate was centrifuged at 40 x *g* for 5 min at 22°C while the other plate remained stationary. Infected cells were then incubated at 37°C 5% CO_2, and washed with PBS at 30 minutes post-infection. For adhesion assays, the coverslips were harvested after the PBS washes. For invasion assays, pre-warmed medium supplemented with gentamicin (150μg/ml) was added after the washes, and coverslips were harvested at 75 min post-infection after one additional PBS wash. Bacterial counts were determined by transferring each coverslip into a 15 ml conical containing 5 ml of sterile water, and plating the lysate on LB agar (18). For each sample, three to five coverslips were used for bacterial counts and one coverslip was stained with DiffQuick. Bacterial counts were also determined for the initial inoculum by plating serial dilutions of the inoculum on LB agar.

Sodium Azide Assay. Assays performed in the presence of sodium azide (NaN_3) were conducted as described for the adhesion assay above, with the following changes. Overnight cultures of bacteria were washed and suspended in antibiotic free tissue culture medium. NaN_3 was added to a concentration of 20mM and the suspension was incubated at room temperature for five minutes. Tissue culture

medium on host cells was replaced with 37°C antibiotic free tissue culture medium with or without 20mM NaN_3 immediately before infection.

Mouse infections. Competitive index assays were performed as described with some modifications (1, 14). Bacteria were grown in BHI with streptomycin at 250 rpm, 30ºC to an OD_{600} of approximately 0.4. Bacteria were centrifuged at 3200 X g, 20ºC, 15 min, washed once with PBS pH 7.1, and suspended to a calculated concentration of $1.6 \text{ X } 10^{10} – 7.0 \text{ X } 10^{10}$ cfu/ml. The two strains being used in the infection were mixed in a 1:1 ratio and cfu were determined by plating serial dilutions onto LB with streptomycin and LB with streptomycin and erythromycin in parallel. Alternatively, the inoculum was plated onto LB plus streptomycin and colonies were individually picked and inoculated onto LB plus streptomycin and erythromycin. Five to eight week old pathogen free female BALB/c mice (Taconic) were fasted for 2 hours before and after gavage. Gavage was performed using a 20G X 1.5 inch animal feeding needle (Popper and sons, inc.) with a dose of $4 \text{ X } 10^9 – 1.75 \text{ X } 10^{10}$ bacteria in a volume of 250 µl. Mice were sacrificed at 4-6, 18 or 42 hours after gavage. Livers were harvested and homogenized in 0.05% NP-40, streptomycin (200 µg/ml), and erythromycin (0.1 µg/ml) to induce the *erm* gene. The entire intestines were removed, cut open longitudinally and contents were flushed out using PBS. Intestines were then placed in homogenization buffer supplemented with 0.2 mM EDTA, incubated for up to one hour at room temperature, and homogenates were plated as described above. Animal protocols were approved by Cornell University Institutional Animal Care and Use Committee and in accordance with all legal requirements.

Statistical analysis. Percent invasion or adhesion was calculated using invasion or adhesion efficiencies (number of internalized or adherent bacteria divided by total

number of bacteria used in infection) normalized to wild-type strain set to 100% for each individual experimental repetition. Invasion and adhesion assays were analyzed using a One-way ANOVA with Bonferroni's multiple comparison test to determine if invasion levels of individual strains were different than wild-type set at 100% or than other strains. The mouse competitive index assay was analyzed with a one-sample Student's *t* test (one-tailed) using log transformed competitive indexes to determine if the indexes were significantly less than one.

RESULTS

Construction and characterization of motility mutants. To assess the role of flagella and flagella-mediated motility in *L. monocytogenes* infection of host cells, an in-frame deletion was made in *flaA*, the gene encoding flagellin, the monomer that makes up the long helical flagellar filament. The mutant is denoted *Lm* Δ*flaA*. Motility phenotypes were confirmed by inoculation into motility agar and observation of wet mounts. As predicted, *Lm* Δ*flaA* mutant was non-motile by wet-mount and produced a compact colony on motility agar plates (Figure 2.1A). The wild-type strain (10403S) was motile and produced a colony with a large growth radius (Figure 2.1A). Bacteria were stained with crystal violet to visualize the flagella. Flagella were not visible at the surface of the *Lm* Δ*flaA* mutant, but were detectable at the surface of the wild-type strain (Figure 2.1B). SDS-extractable bacterial surface proteins were examined by Western immunoblot using an antibody to *L. monocytogenes* flagella. As expected, flagellin was detected from the wild-type strain but not from *Lm* Δ*flaA* (Figure 2.1C). Additionally, we constructed a complemented *Lm* Δ*flaA* strain (*Lm* Δ*flaA flaA*$^{+}$) by inserting the *flaA* open reading frame into the tRNA^{arg} site on the

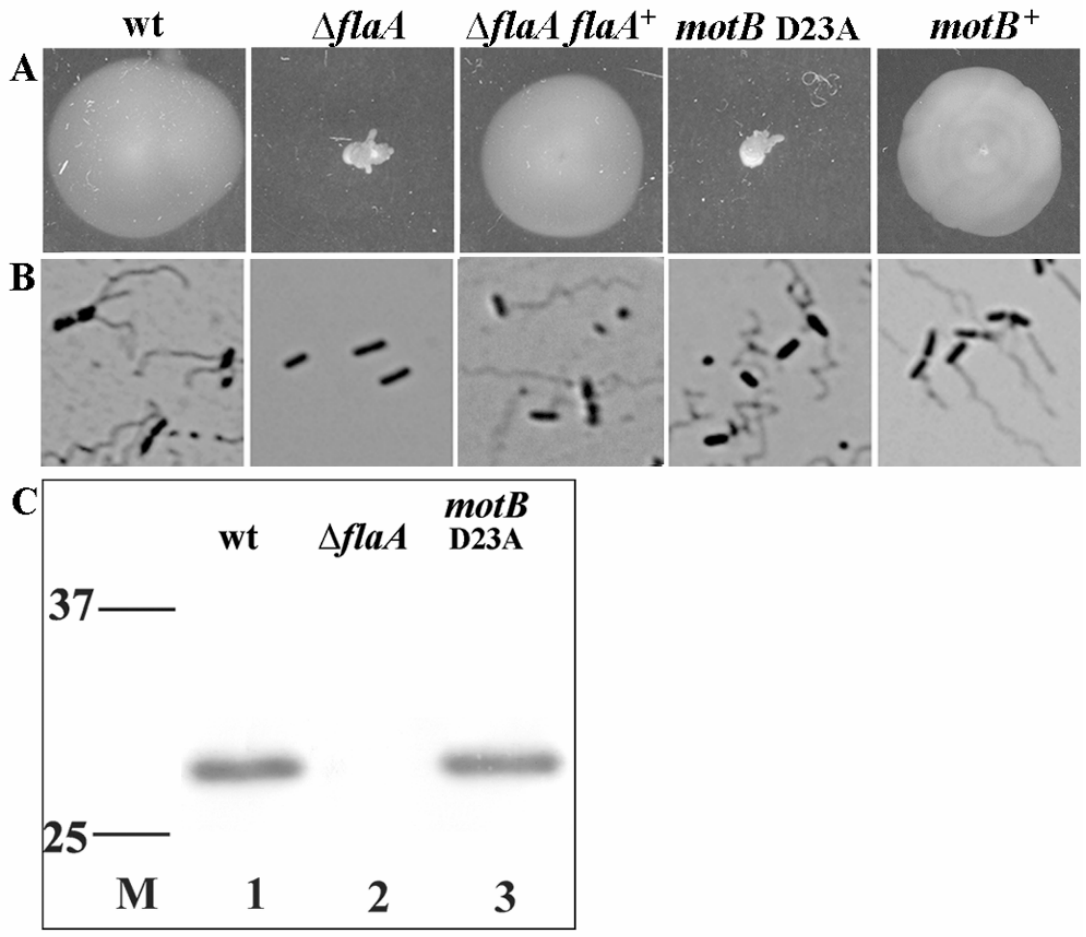

Figure. 2.1. Characteristics of the wild-type and motility mutant strains.

A. Detection of motility: Strains of *L. monocytogenes* were stabbed into soft LB agar and incubated at room temperature for 36 hours. A large area of bacterial growth is indicative of bacterial motility. B. Detection of bacteria-associated flagella: Bacteria grown in BHI at 30°C shaking were stained with crystal violet to visualize flagella by bright field microscopy. C. Immunodetection of flagellin from *L. monocytogenes*. Bacterial surface extracted proteins were resolved by SDS-PAGE and flagellin was detected by Western immunoblot. Lane M: pre-stained markers with molecular mass indicated in kDa on the left.

chromosome. This complemented strain was flagellated (Figure 2.1B) and motile (Figure 2.1A).

To directly determine if the flagellar filament is used as an adhesin, we needed to construct a flagellated but non-motile mutant. This was initially attempted by creating an in-frame deletion in *motB*, a gene encoding one of the flagellar motor proteins, creating *Lm* Δ*motB*. The *Lm* Δ*motB* mutant was non-motile by wet mount and motility agar. However, although flagella were detectable at the surface of the *Lm* Δ*motB* mutant, 55% or less of the bacteria were flagellated and flagellated bacteria appeared to carry fewer flagella than wild-type bacteria when examined by crystal violet staining. Additionally, by western immunoblot, the *Lm* Δ*motB* mutant appeared to have considerably less flagellin than the wild-type strain. We rationalized that MotB is structurally important for assembly of the flagellar apparatus and therefore created a different mutant preserving MotB structure. This mutant, *Lm motB*D23A, contains an alanine in position 23 of the MotB open reading frame instead of an aspartic acid. The corresponding aspartic acid in *E.coli* has been shown to be essential for torque generation (29). This mutant was non-motile by wet mount and motility agar (Figure 2.1A), and fully flagellated by crystal violet staining (Figure 2.1B) and western immunoblot (Figure 2.1C). The *Lm motB*D23A mutant is therefore a fully flagellated but non-motile strain and an ideal tool for addressing the question of flagellar filaments as adhesins. The *Lm motB*D23A mutant was complemented by inserting the wild-type gene into the chromosome by allelic exchange. The complemented strain, *Lm motB*$^{+}$ was flagellated, and motile by wet mount and motility agar (Fiugre 2.1A and B).

Functional flagella are required for optimal invasion of human intestinal epithelial cells by *L. monocytogenes*. The human intestinal epithelial cell line Caco-2 was used to evaluate the invasion phenotypes of *L. monocytogenes* motility mutants. First, as previously shown by other groups, we observed that the *Lm* Δ*flaA* mutant is significantly less invasive than the wild-type strain, but has no defect in intracellular growth and cell-to-cell spread (6, 27) (Table 2.3 and data not shown). Similarly, we confirmed that centrifugation of non-motile non-flagellated bacteria onto host cells only partially complements the invasion defect, indicating that flagella contribute more to invasion than a mere increase in the probability of bacteria-host cell contact (6) (Table 2.3). The complemented *Lm* Δ*flaA flaA*$^{+}$ strain did not invade Caco-2 cells to the same level as wild-type strain, but this difference was not significant (Table 2.3). This incomplete complementation may be caused by the exclusion of the recently described MogR binding promoter region III (22) and the integration of the *flaA* promoter and ORF sequences at a new location on the chromosome.

Next, to determine whether the flagellar filament per se contributes to *L. monocytogenes* ability to invade epithelial cells independent of motility, we tested the flagellated but non-motile *Lm motB*D23A mutant. Results indicate that the flagellated but non-motile *Lm motB*D23A strain was as deficient as the non-flagellated non-motile *Lm* Δ*flaA* strain, suggesting that non-motile flagella do not contribute to invasion of epithelial cells by *L. monocytogenes* (Table 2.3). Similarly to what we observed for the *Lm* Δ*flaA* strain, centrifugation of *Lm motB*D23A strain onto host cells only partially complemented the invasion defect. The complemented strain *Lm motB*$^{+}$ behaved like the wild-type strain.

Table 2.3. *L. monocytogenes* adhesion to and invasion of Caco-2 cells

Strain	Treatment With: Centrifugation	NaN_3	% adhesion[a](n)	% invasion[a](n)
Δ*flaA*	-	-	38.25 ± 9.01*[b] (3)	0.71 ± 0.31**(4)
Δ*flaA*	+	-	88.31 ± 38.26 (3)	16.73 ± 8.99**(4)
Δ*flaA flaA*$^+$	-	-	ND[c]	58.76 ± 30.14(5)
*motB*D23A	-	-	3.86 ± 0.94** (3)	0.32 ± 0.22**(4)
*motB*D23A	+	-	18.18 ± 5.39** (**)(3)	3.68 ± 1.85**(4)
motB$^+$	-	-	95.41 ± 26.39 (3)	153.10 ± 51.38(4)
wt	-	+	7.94 ± 0.40**(3)	ND
*motD*23A	-	+	9.15 ± 7.76**(3)	ND

[a] % of wild-type ± SD. Absolute adhesion levels for wild type strain were $9.39 \times 10^{-4} \pm 1.69 \times 10^{-4}$ and $3.58 \times 10^{-3} \pm 8.72 \times 10^{-4}$ without and with centrifugation, respectively. Absolute invasion levels for wild type strain were $2.67 \times 10^{-4} \pm 1.62 \times 10^{-4}$ and $1.78 \times 10^{-3} \pm 1.38 \times 10^{-3}$ without and with centrifugation, respectively.

[b] *P* values (*, < 0.01; **, < 0.001) for comparison by one-way ANOVA with Bonferroni's multiple comparison test of adhesion and invasion capabilities between a respective strain and the wild-type strain. In parentheses are the *P* values for comparison of the following pairs of strains: *Lm* Δ*flaA* and *Lm motB*D23A without centrifugation, *Lm* Δ*flaA* and *Lm motB*D23A with centrifugation, wild-type and *Lm motB*D23A treated with NaN_3, *Lm motB*D23A not treated and treated with NaN_3. The absence of an asterisk indicates a non-significant *P* value.

[c] ND, not determined

The flagellar filament does not improve adhesion of *L. monocytogenes* to cultured human intestinal epithelial cells. Adhesion assays were performed with the *Lm* Δ*flaA* and *Lm motB*D23A mutants to determine whether the flagellar filament contributes independently of motility to the ability of *L. monocytogenes* to adhere to epithelial cells. Semi-confluent monolayers of Caco-2 cells were infected for 30 minutes and washed before determining counts of cell-associated bacteria. Results indicate that flagellated non-motile and non-flagellated bacteria were significantly deficient in their ability to adhere to Caco-2 cells (Table 2.3). The adhesion rate of *Lm* Δ*flaA* strain was not significantly different than that of wild-type strain after centrifugation, whereas that of *Lm motB*D23A strain was. Moreover, the levels of adhesion of *Lm* Δ*flaA* and *Lm motB*D23A strains were significantly different from each other after centrifugation indicating that non-motile flagella interfere with adhesion of *L. monocytogenes* to epithelial cells. The adhesion rate of the complemented strain, *Lm motB*$^{+}$, was equivalent to that of wild-type strain.

Alternatively, we used an energy poison to determine whether the flagellar filament contributes independently of motility to the ability of *L. monocytogenes* to adhere to epithelial cells. Conditions for this experiment were optimized by incubating bacteria for five minutes in various concentrations of the ATPase inhibitor NaN_3, and checking for motility by wet mount. At 20mM NaN_3 very few bacteria remained motile. This concentration of NaN_3 was non-lethal to *L. monocytogenes* within the time frame of the assay as determined by bacterial counts before and after a 30-minute treatment. The adhesion assay consisted of comparing motile wild-type bacteria to NaN_3 treated non-motile wild-type bacteria. As a control for the effect of NaN_3 on bacterial adhesion, we tested the adhesion levels of flagellated but non-motile *Lm motB*D23A bacteria with NaN_3 present. The addition of NaN_3 brought wild-type adhesion levels

down to the levels observed with *Lm motB*D23A mutant (Table 2.3). Adhesion levels of NaN_3 treated wild-type and *Lm motB*D23A strains as well as those of untreated and treated *Lm motB*D23A mutant to host cells were not significantly different from each other. Together, these data show that the presence of non-motile flagella is not beneficial for *L. monocytogenes* adhesion to host cells, indicating that the flagellar filament does not function as an adhesin.

Motile bacteria out compete non-motile bacteria in initial colonization of the mouse intestines and liver. The previous assays clearly demonstrate that flagella-mediated motility contributes significantly to the ability of *L. monocytogenes* to invade epithelial cells. To assess the importance of flagella-mediated motility for early colonization of the intestines, a competitive index (CI) assay was performed by orally infecting 5-8 week-old female BALB/c mice with a 1:1 mixture of strain DP-L3903, an erythromycin-resistant derivative of 10403S that is fully virulent, and the isogenic *Lm* Δ*flaA* mutant strain. At 4-6 hours post-infection, the *Lm* Δ*flaA* mutant showed a 2.2 fold decrease in colonization of the mouse intestines (CI of 0.46; $P < 0.001$) (Figure 2.2A). However, by 18 hours post-infection this attenuation was no longer detectable. At 18 hours post-infection very few mice had detectable numbers of bacteria in their liver, but the colonization deficiency observed in the intestines was mimicked in the liver of infected mice, with a 4.1 fold decrease in colonization (CI of 0.24; $P = 0.011$) (Figure 2.2B). This deficiency was no longer present by 42 hours post-infection (CI of 0.930) (Figure 2.2B). The complemented strain *Lm* Δ*flaA flaA*$^+$ was not deficient in colonization of the intestines or liver when compared to the wild-type strain (Figure 2.2A and B).

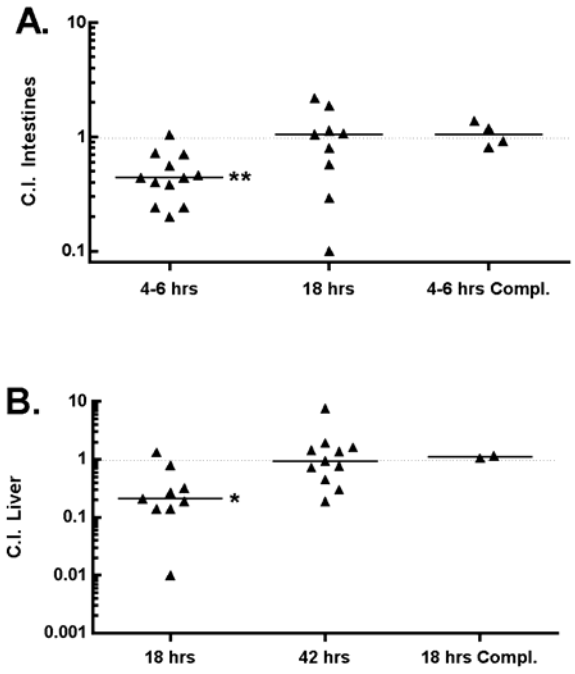

Figure 2.2. Motile bacteria out compete non-motile bacteria in initial colonization of the murine intestines and liver. Mice were infected orally with a 1:1 ratio of 10403S *erm*$^+$ and *Lm* Δ*flaA*, 10403S and *Lm* Δ*flaA erm*$^+$, or 10403S *erm*$^+$ and *Lm* Δ*flaA flaA*$^+$ as described in detail in Materials and Methods. Competitive indexes (CI) were determined for colonization of the intestines (A) and liver (B) at indicated time points post-infection. Each data point represents the CI of one mouse. Data points were collected from a minimum of two independent experiments, except for the complemented *Lm* Δ*flaA* mutant strain for which a single experiment was performed. A CI value of less than one indicates that the mutant strain was out competed by the wild-type strain. No differences were observed when mice were infected with 10403S *erm*$^+$ and *Lm* Δ*flaA* versus 10403S and *Lm* Δ*flaA erm*$^+$, indicating that the erythromycin-resistance gene did not influence CI results. Horizontal bars indicate the median. Statistical differences from 1.0 were determined by Student's *t* test: * indicates $P < 0.05$ and **indicates $P \leq 0.005$.

To ensure that the erythromycin resistance cassette did not cause the phenotypes by providing an advantage to DP-L3903, the experiment was also conducted using 10403S and Hel-487, a *Lm* Δ*flaA* strain transduced with the erythromycin cassette from DP-L3903. We observed that the CI were not influenced by the erythromycin cassette. Overall, these results indicate that flagella-mediated motility influences *L. monocytogenes* infectivity in mice at an early time point after oral infection.

DISCUSSION

Flagella and flagella-mediated motility are integral to the virulence of several gastrointestinal bacterial pathogens (10). For *L. monocytogenes*, no link has been made between flagella and virulence, although the flagella is important for efficient invasion of tissue culture cells (2, 6). In this study, we investigated the mechanism by which flagella influence the ability of *L. monocytogenes* to invade host cells and the role of flagella in colonizing mice early in infection. Our results clearly indicate that *L. monocytogenes* flagella do not function as adhesins to enhance bacterial attachment to and invasion of epithelial cells, but rather function as motility devices contributing more to invasion that a mere increase in probability of bacteria-host cell interaction. Moreover, we show that motile bacteria out compete non-motile bacteria for initial colonization of the intestinal tract and liver by *L. monocytogenes*.

Flagella can function as adhesins, independent of motility, to enhance bacterial invasion of host cells as shown for enteropathogenic *E. coli* (7). For *L. monocytogenes*, the presence of flagella increases bacterial adherence to host cells (2, 6), but it is not known whether or not the flagellum itself serves as an adhesin. The present study addressed this question directly by comparing the behavior of flagellated

motile (wild-type), flagellated non-motile (*Lm motB*D23A) and non-flagellated bacteria (*Lm* Δ*flaA*), as well as that of flagellated motile (wild-type) and non-motile bacteria (wild-type treated with NaN_3). In both instances, the outcome indicated that flagella do not serve as adhesins for attachment of *L. monocytogenes* to host cells. Moreover, the fact that flagellated non-motile bacteria (*Lm motB*D23A) attach to host cells less efficiently than non-flagellated bacteria (*Lm* Δ*flaA*) also indicated that non-motile flagellar filaments interfere with *L. monocytogenes* adhesion to host cells. This could be due to the non-motile filaments preventing direct bacterial cell surface interactions with host cell receptors. Similarly, wild-type bacteria attached less efficiently to host cells when motility was inhibited with an energy poison. Therefore, *L. monocytogenes* peritrichous flagella function as motility devices and not as adhesins to enhance host cell invasion.

Analysis of the efficacy of adhesion relative to that of invasion reveals that flagella-mediated motility influences *L. monocytogenes* invasion of epithelial cells to a much larger extent than adhesion. When absolute adhesion and invasion numbers are taken into consideration, the invasion level of wild-type strain was approximately 3 fold lower than its adhesion level, however, the invasion levels of *Lm* Δ*flaA* and *Lm motB*D23A mutants were more than 100 fold lower than adhesion levels. When bacteria were centrifuged onto host cells, the invasion level of wild-type strain was approximately 2 fold lower than its adhesion level, whereas invasion levels of *Lm* Δ*flaA* and *Lm motB*D23A mutants were more than 10 fold lower than adhesion levels. These observations suggest that flagella-mediated motility contributes more to invasion of epithelial cells than a mere increase in the probability of bacteria-host cell contact. Perhaps flagella-mediated motility is required for *L. monocytogenes* to maintain contact with host cells until high affinity ligand-receptor binding has been

established. It is conceivable that the non-motile mutants are unable to sustain contact with host cells long enough to ensure this type of interaction. It is also possible that the physical force generated by flagella-mediated motility against the host cell membrane contributes to invasion in a manner similar to intercellular spread of bacteria that use an actin-based mechanism of motility (15). Consistent with this hypothesis, physical force generated by centrifuging non-motile bacteria unto host cells improved invasion although not to wild-type levels. Moreover, flagella-mediated motility may be required for proper spatial orientation of bacteria upon attachment to host cells. Non-motile *L. monocytogenes* mutants are less defective at invading professional phagocytic cells than epithelial cells as the invasion of J774 mouse macrophage cells by *Lm* Δ*flaA* was 48.4 ±13.6 % of wild type (6). Professional phagocytic cells are equipped with cell-surface receptors that recognize common Gram-positive surface molecules (24). The only limitation for *L. monocytogenes* invasion of professional phagocytes may be the probability of bacteria-host cell interaction, since ligands are distributed evenly on the bacterial surface and ligand-receptor interactions lead to rapid phagocytosis. On the contrary, invasion of epithelial cells by *L. monocytogenes* requires the presence of specialized invasins at the bacterial surface, which may have a polar distribution as recently shown for InlA (19). Efficient engagement of InlA with its receptor, E-cadherin, would require that bacteria interact head on with host cells. The requirement for spatial orientation could also explain why centrifuging non-motile *L. monocytogenes* onto host cells does not rescue the invasion deficiency to wild-type level (Table 2.3) (6). Overall, it appears that the contribution of flagella-mediated motility to host cell invasion possibly encompasses mechanisms related to probability of host cell contact, physical force, and spatial orientation.

The fact that no association has been made between flagella-mediated motility and *in vivo* virulence is surprising considering the major invasion defect observed in tissue culture epithelial cells (2, 6, 27). We rationalized that if flagella-mediated motility contributes to infection *in vivo* it would be, as for other foodborne pathogens, at the level of intestinal colonization early after oral infection. The ability of the non-motile mutant (*Lm* Δ*flaA*) to colonize the intestines of mice was tested in a competitive assay with the wild-type strain. The competitive index assay is more statistically powerful than comparing bacterial counts between mice because it eliminates mouse-to-mouse variation. Additionally, competitive index experiments can elucidate differences in bacterial fitness due to the added element of competition. In that context, the non-motile *L. monocytogenes* strain was deficient (2.2 fold) in colonization of the mouse intestines at 4-6 hours post-infection. Moreover, this deficiency was reflected in colonization of the liver (4.1 fold) twelve hours later, consistent with the liver being directly connected to the intestines through the portal circulation system. These observations were not made in previous studies presumably because colonization of the intestines and liver was not evaluated at these early time points post-infection (2, 6). However, in agreement with these previous studies, differences in colonization of the intestines and liver by motile and non-motile bacteria were no longer detected at later time points. It has been hypothesized that, in mice, *L. monocytogenes* invasion occurs through Peyer's patches rather than through the intestinal epithelium as the major *L. monocytogenes* invasin, InlA, does not bind the mouse E-cadherin (13). Considering that flagella-mediated motility is far less important for infection of phagocytic cells than epithelial cells (2), its importance for *L. monocytogenes* virulence is likely to be underestimated in a mouse model. Perhaps, in a susceptible human, the contribution of flagella-mediated motility on *L. monocytogenes* ability to colonize the intestinal tract would have a greater impact on the outcome of infection.

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CHAPTER 3

THE PROPEPTIDE OF THE METALLOPROTEASE OF *LISTERIA MONOCYTOGENES* CONTROLS COMPARTMENTALIZATION OF THE ZYMOGEN DURING INTRACELLULAR INFECTION.*

* Heather S. O'Neil, Brian M. Forster, Kari L. Roberts, Andrew J. Chambers, Alan Pavinski Bitar and Hélène Marquis. The propeptide of the metalloprotease of *Listeria monocytogenes* controls compartmentalization of the zymogen during intracellular infection. Currently in submission to Journal of Bacteriology.

ABSTRACT

Integral to the virulence of the intracellular bacterial pathogen *Listeria monocytogenes* is the metalloprotease (Mpl). Mpl regulates the activity and compartmentalization of the bacterial broad-range phospholipase C (PC-PLC). Mpl is secreted as a proprotein that undergoes intramolecular autocatalysis to release the catalytic domain. In related proteases, the propeptide serves as a folding catalyst and can act either *in cis* or *in trans*. Propeptides can also influence protein compartmentalization, intracellular trafficking, or decrease folding kinetics. In this study, we aimed to determine the role of the Mpl propeptide by monitoring the behavior of Mpl synthesized in absence of its propeptide (MplΔpro) and of two Mpl single site mutants with unstable propeptides (Mpl H75V and Mpl H95L). We observed that all three Mpl mutants mediate PC-PLC activation when bacteria were grown on semi-solid medium. However, the mutant proteins were not functional in infected cells as determined by monitoring PC-PLC maturation and compartmentalization. This defect could not be rescued by providing the propeptide *in trans* to the *mpl*Δpro mutant. We also determined that PC-PLC co-purifies with wild-type Mpl, MplΔpro, and the Mpl propeptide indicating that the propeptide is not required for Mpl to interact with PC-PLC. Last, we tested the compartmentalization of Mpl during intracellular infection and observed that the mutant Mpl species were aberrantly secreted in the cytosol of infected cells. These data indicated that the propeptide of Mpl serves to maintain Mpl bacteria-associated, and that this localization is essential to the function of Mpl during intracellular infection.

INTRODUCTION

Listeria monocytogenes is a Gram-positive facultative intracellular pathogen and the causative agent of the food-borne disease listeriosis in humans and a variety of vertebrates (41). The success of *L. monocytogenes* as a pathogen can be attributed largely to its ability to replicate in the host cell cytosol and to spread from cell to cell without entering the extracellular milieu (10, 39). Efficacy of escape from vacuoles formed upon initial entry into a host cell or upon cell-to-cell spread is imperative to the virulence of *L. monocytogenes* (31, 39, 40).

There are multiple bacterial factors involved in vacuolar escape including the broad range phospholipase C, PC-PLC (31, 40). PC-PLC is synthesized as an inactive proenzyme, whose maturation into an active form requires proteolytic cleavage of a *N*-terminal propeptide (23). During infection, PC-PLC is stored as a proenzyme at the interface of the bacterial membrane and cell wall (19, 33), and is released as a bolus of mature protein upon a drop in pH such as is experienced in the host vacuole (18, 19). Maturation and translocation of PC-PLC across the cell wall are dependent on a decrease in pH and on the activity of the zinc metalloprotease of *Listeria*, Mpl (18, 19, 24, 25).

ActA, a *L. monocytogenes* surface protein, also serves as a substrate for Mpl. Similar to PC-PLC, ActA is cleaved by Mpl upon a decrease in pH (26). ActA mediates the polymerization of host actin filaments on the bacterial surface to generate bacterial movement in the cytosol (13). Presumably, cleavage of ActA upon exit from the spreading vacuole enables bacterial replication before actin-based movement resumes (26).

Mpl is a member of the M4 family of metalloproteases, represented by thermolysin from *Bacillus thermoproteolyticus* (22). Mpl is translated as a preproenzyme with a 24 amino acid signal sequence that is removed upon secretion across the bacterial membrane (21). The secreted Mpl zymogen matures exclusively by intramolecular autocatalysis releasing a 176 amino acid propeptide and a 310 amino acid mature protease (5).

Many proteases are synthesized as preproenzymes similar to Mpl. Propeptides often function as intramolecular chaperones catalyzing the folding of their covalently bound protease (30). However, many propeptides remain functional as folding catalysts when added *in trans* (17, 20, 37, 45). This folding process is best studied in the serine proteases subtilisin and α-lytic protease (34, 36, 43). The propeptide guides folding of the catalytic domain through a non-native folding intermediate to an unprocessed native-fold by lowering the free-energy required to achieve the native state (1, 27, 34). This is rapidly followed by autocatalysis to cleave off the propeptide. The propeptide is retained in a stable propeptide:protease complex, and serves as an inhibitor (2, 35, 43). The rate determining step in production of active protease is the degradation of the inhibitory propeptide (36). Degradation of the propeptide by its cognate protease releases the active enzyme, increasing the energy barrier and preventing unfolding (34).

Propeptides can serve functions other than as a folding catalyst. Targeting of the pepsin-like aspartic protease Cathepsin D to the lysosome requires the propeptide and deletion of the propeptide prevents export of the protease from the endoplasmic reticulum (ER) (9, 38). Secretion of the cell surface metalloprotease ADAMTS9 depends on a covalently bonded propeptide that is properly glycosylated. This

propeptide is cleaved at the cell surface by furin then remains associated with the protease until the complex is released from the cell surface (14). The propeptide of human myeloperoxidase is responsible for increasing the amount of time the protein spends in the ER, probably to aid in heme incorporation, a requirement for further processing (11). The *in vitro* refolding of denatured lipase from *Rhizopus oryzae* is slowed by the presence of its propeptide (3). Finally, the propeptide of *L. monocytogenes* PC-PLC serves to retard translocation of the protein across the cell wall, enabling the protein to accumulate at the membrane cell wall interface until it is released as a bolus to mediate bacterial escape from vacuoles formed during cell-to-cell spread (44).

The present study examines the role of the Mpl propeptide. Our results indicate that the propeptide of Mpl is not essential for activity. However, the propeptide serves to retain Mpl bacteria-associated, and the compartmentalization of Mpl is integral to its ability to mediate PC-PLC maturation and ActA proteolysis during intracellular infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. monocytogenes* strains used in this study are listed in Table 3.1 and plasmids are listed in Table 3.2. *L. monocytogenes* was routinely cultured in brain heart infusion broth (BHI). *E. coli* DH5-α strains carrying pKSV7 (32) derived plasmids were cultured in Luria Bertani (LB) broth supplemented with ampicillin (100 μg/ml), whereas *E. coli* strains carrying pAM401 (42) were cultured in LB supplemented with chloramphenicol (10 μg /ml). *L. monocytogenes* strains carrying pKSV7 or pAM401 were cultured in BHI

Table 3.1 *L. monocytogenes* strains used in this study

Strain	Genotype and Relevant Features	Source/Ref
10403S	Wild type (serotype 1/2a)	(4)
NF-L943	*prfA* G155S in 10403S background. Overexpresses PrfA-dependent genes including *mpl* and *plcB*	(29)
DP-L1935	Internal in frame deletion of *plcB* in 10403S background	(31)
DP-L2343	Deletion of *mpl* structural gene in 10403S background	(28)
HEL-469	Internal in frame deletion of *mpl* in NF-L943 background	(5)
HEL-780	m*pl* H95L in 10403s background	This study
HEL-782	*mpl* H95L in NF-L943 background	This study
HEL-784	*mpl* H75V in 10403S background	This study
HEL-786	*mpl* H75V in NF-L943 background	This study
HEL-798	*mpl*-Flag in NF-L943 background	(5)
HEL-871	Deletion of *mpl* propeptide in 10403S background	This study
HEL-903	10403S + pAM401	This study
HEL-904	HEL-871 + pAM401	This study
HEL-905	DP-L2343 + pAM401	This study
HEL-907	DP-L1935 + pAM401	This study
HEL-908	HEL-871 + *mpl* propeptide *in trans* (pHSO909)	This study
HEL-925	Internal inframe deletion of *plcB* in NF-L943 background	This study
HEL-927	Deletion of *mpl* propeptide in NF-L943 background	This study
HEL-938	HEL-927 + *mpl* propeptide *in trans* (pHSO909)	This study
HEL-939	HEL-925 + pAM401	This study
HEL-940	NF-L943 + pAM401	This study
HEL-941	HEL-469 + pAM401	This study
HEL-942	HEL-927 + pAM401	This study
HEL-943	*mpl*Δpro-Flag in NF-L943 background	This study
HEL-974	*mpl* propeptide-Flag in HEL-469 background	This study

Table 3.2 Plasmids used in this study

Plasmid	Relevant Features	Source/Ref
DP-1888	pKSV7Δ*plcB* for internal inframe deletion	(31)
pAB796	pKSV7 *mpl* C-terminus plus Flag tag	(5)
pAM401	Shuttle vector	(42)
pHSO849	pKSV7 *mpl*Δpro	This study
pHSO890	pAM401 with spac promoter	This study
pHSO909	pHSO890 with *mpl* signal sequence and propeptide	This study
pHSO973	pAM401 *mpl* 5' UTR, signal sequence, and propeptide-Flag	This study
pKR778	pKSV7 *mpl*H95L	This study
pKR779	pKSV7 *mpl*H75V	This study
pKSV7	Shuttle vector for allelic exchange	(32)

supplemented with chloramphenicol (10 μg/ml). In preparation for intracellular immunoprecipitation assays and immunofluorescence, *L. monocytogenes* was cultured overnight in BHI broth with or without chloramphenicol (10 μg/ml) as appropriate, 30ºC without shaking. Cultures used for Western immunoblots were grown in LB with 50 mM morpholinepropanesulfonic acid (MOPS), adjusted to pH 7.3 and supplemented with 0.2% activated charcoal, 25 mM glucose (LB-MOPS-Glc) (33) and chloramphenicol (10 μg/ml) if appropriate.

Construction of in-frame deletion mutants. A 528 bp gene segment coding for the propeptide of Mpl was deleted to create *mpl*Δpro by site-directed mutagenesis with overlap extension (SOEing) (12). Two sets of primers were designed to amplify DNA from regions upstream and downstream of the segment to be deleted. Amplification from *L. monocytogenes* 10403S chromosomal DNA of a 503 bp region encompassing the *mpl* 5' untranslated region (UTR), promoter region, and open reading frame (ORF) coding for the signal sequence was performed by PCR using forward primer Marq344 and reverse primer Marq345 (Table 3.3). A 368 bp region coding for the N-terminal of the Mpl mature form was also amplified by PCR using forward primer Marq346 and reverse primer Marq347. These two PCR products were used in a SOEing PCR with primers Marq344 and Marq347. The resulting 824 bp product was digested with KpnI and EcoRI, ligated into the shuttle vector pKSV7, and transformed into DH5-α creating plasmid pHSO849. The cloned fragment was sequenced to ensure accuracy and the plasmid was electroporated into 10403S and NF-L943 (29) to replace the wild type *mpl* allele with *mpl*Δpro by allelic exchange (7) generating strain HEL-871 and HEL-927 respectively. Mutants were identified by PCR amplification of an 824 bp fragment with primers Marq344 and Marq347 from chloramphenicol sensitive clones.

Table 3.3. Oligonucleotide primers used in this study

Primer	5'→3' sequence[a]	Characteristics
Marq 313	TAACTGCAGCATATCCCAAAGTTTAAGC	PstI
Marq 314	CGGTGTGACAAGGACCTTGATT	Eco0109I
Marq 315	AATCAAGGTCCTTGTCACACCG	Eco0109I
Marq 316	TAGAGCTCCACTGCGGAACTAAATTCTG	SacI
Marq 317	CGAGCGTATACACCGTAACGCCTA	Bst1107I
Marq 318	TAGGCGTTACGGTGTATACGCTCG	Bst1107I
Marq 344	GCGGTACCAATAGAAATATCTCCATCTGG	KpnI
Marq 345	CCATAGTCGGGCAAGATGTGCTTTTACCGTCATAGTGA	
Marq 346	TCACTATGACGGTAAAAGCAGTAGAACGGGCTGATACC	
Marq 347	GCGAATTCCCATAATGGACAAACGAATC	EcoRI
Marq 395	GAGCATGCAGAGGAGTTTTATGAAAAGTAAACTTATTTGTATC	SphI
Marq 396	CTGCATGCCTCACTCGGAAAGCATATTTTGCTT	SphI
Marq 398	GCGGATCCCTAACAGCACAAGAGCGGAAAG	BamHI
Marq 399	GAGCATGCTCACCTCCTTAAGC	SphI
Marq 400	GAGCATGCAGAAGAATTAACAAATGTAAAAG	SphI
Marq 484	CTGCATGCTCACTTGTCATCGTCATCCTTGTAATCCTCGAGCTCG	
	GAAAGCATATTTTGCTT	SphI/Flag

[a] Restriction sites and the Flag Tag are underlined

A 276 bp internal in-frame deletion of *plcB*, the gene coding for PC-PLC, was generated by allelic exchange in NF-L943 using the pKSV7 based plasmid DP-1888 (31), generating strain HEL-925. Plasmid DP-1888 was originally used to create DP-L1935, a 10403S derivative with an internal in-frame deletion in *plcB*. Chloramphenicol sensitive colonies were screened for the absence of PC-PLC activity on egg-yolk agar plates as described below.

Strains were electroporated with the vector pAM401 as needed and selected for resistance to chloramphenicol (10 μg/ml).

Construction of *mpl* point mutants. Point mutations in the *mpl* gene were generated by SOEing PCR as described above. The histidine residue at position 75 was substituted with a valine residue using primer pairs Marq313/Marq314, and Marq315/Marq316. Marq314 and Marq 315 provide the necessary codon change as well as a silent mutation to create a novel Eco0109I restriction site for screening. A final PCR product of 1002 bp was digested with PstI and SacI, ligated into the shuttle vector pKSV7 and transformed into DH5-α creating plasmid pKR779. The histidine residue at position 95 was substituted with a leucine residue using primer pairs Marq313/Marq317, and Marq318/Marq316. Marq317 and Marq318 provide the necessary codon change and a silent mutation to generate a Bst1107I restriction site for screening. A final PCR product of 1002 bp was digested with PstI and SacI, ligated into the shuttle vector pKSV7 and transformed into DH5-α creating plasmid pKR778. Point mutations and fidelity of sequence were verified by sequencing before electroporating the plasmids into 10403S and NF-L943 to replace the wild-type *mpl* allele by allelic exchange. Mutants were screened for chloramphenicol sensitivity and acquisition of a novel Eco01091 restriction site for the H75V mutation or Bst1107I

restriction site for H95L mutation. The *mpl* H75V mutant strains generated were identified as HEL-784 (10403S background) and HEL-786 (NF-L943 backgound). The *mpl* H95L mutant strains generated were identified as HEL-780 (10403S background) and HEL-782 (NF-L943 backgound).

Mpl propeptide complementation *in trans*. The *mpl*Δpro strains HEL-871 and HEL-927 were complemented *in trans* by cloning the *mpl* gene fragment coding for the signal sequence and propeptide downstream of a spac promoter on a multicopy plasmid. The spac promoter was amplified from pLiv (8) using Marq398 and Marq399. The product was digested with BamHI and SphI, ligated into pAM401, sequenced and named pHSO890. The sequence coding for *mpl* signal sequence and propeptide was amplified from 10403S genomic DNA using Marq395 and Marq396. This 622 bp product was digested with SphI and ligated into pHSO890 creating pHSO909. Chloramphenicol resistant colonies were screened by PCR using Marq398 and Marq396 to determine if the insert was present and in the proper orientation. Cloned constructs were verified by sequencing then electroporated into HEL-871 and HEL-927. Chloramphenicol resistant colonies were selected generating HEL-908 and HEL-938.

Western immunoblotting. *L. monocytogenes* strains with a NF-L943 background were used for these experiments. Bacterial cultures grown to an OD_{600} of ~1.0 were chilled on ice, centrifuged, and supernatants were collected. Trichloroacetic acid (TCA) was added to a final concentration of 5% and supernatants were incubated on ice for one hour. Precipitated proteins were washed with acetone, dissolved in 2X sample buffer (125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate [SDS], 20% glycerol, 20 mM DTT), and heat-denatured for 5 minutes. Extracted proteins

equivalent to 1.0 ml of culture with an OD_{600} of 1.0 were resolved on 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane using a semidry electroblotting apparatus. The protein blot was reacted with rabbit immune serum to *L. monocytogenes* Mpl (kindly provided by Daniel Portnoy) at a dilution of 1/1000 followed by goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (24 ng/ml) (Jackson ImmunoResearch Laboratories, Inc.). Enzymatic reactivity was detected with nitroblue tetrazolium (0.33 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.17 mg/ml).

Phospholipase activity egg yolk agar. *L. monocytogenes* strains with a NF-L943 background were used for these experiments. PC-PLC activity was detected as previously described on LB-egg yolk agar supplemented with or without chloramphenicol at a final concentration of 10 μg/ml as appropriate (18).

Metabolic labeling and immunoprecipitation experiments. *L. monocytogenes* strains with a 10403S background were used for metabolic labeling and immunoprecipitation experiments. Briefly, infected mouse macrophage-like J774 cells were pulse-labeled with [^{35}S]methionine in the presence of host protein synthesis and proteasome inhibitors as described previously (33, 44). For detection of ActA and PC-PLC, bacteria cell-to-cell spread was blocked with cytochalasin D prior to metabolic labeling, and the intracellular pH of labeled infected cells was modified during a chase period by incubating the cells in a potassium-based buffer supplemented with nigericin. For detection of ActA, pulse-chased infected cells were lysed in warm 2X sample buffer, incubated at 100°C for 5 minutes, frozen quickly on dry ice, heat treated for an additional 5 minutes, then resolved on a 10% SDS-PAGE gel. For immunoprecipitation of PC-PLC, infected cells were lyzed in NP-40 buffer

(33) and lysates were centrifuged to pellet bacteria. Bacteria were lyzed using a *Listeria*-specific cell wall hydrolase (15) . PC-PLC was immunoprecipitated independently from host cell and bacterial lysates using affinity-purified rabbit antibodies as described previously (33). For immunoprecipitation of Mpl, samples were processed as described above for PC-PLC except that cells were pulse-labeled for 30 minutes and Mpl was immunoprecipitated from host cell lysates only using an anti-Mpl rabbit polyclonal serum. Labeled proteins were detected by autoradiography.

Addition of a C-terminal Flag tag to Mpl species. A Flag tag sequence was fused in frame to the 3' end of *mpl*Δpro in strain HEL-927 by allelic exchange using the pKSV7 based shuttle vector pAB796 (5), generating strain HEL-943. Another construct was made by fusing a Flag tag sequence to the 3' end of the *mpl* propeptide sequence. This construct was generated by amplifying, from *L. monocytogenes* 10403S chromosomal DNA, a 854 bp region encompassing the *mpl* 5' UTR, promoter region, signal sequence, and propeptide using forward primer Marq 400 and reverse primer Marq 484. This PCR product was digested with SphI, ligated into pAM401 then transformed into DH5-α creating plasmid pHSO973. The cloned construct was verified by sequencing. pHSO973 was electroporated into the *mpl* deletion mutant, HEL-469, and selected for by chloramphenicol resistance, generating HEL-974.

Co-immunoprecipitation experiments. *L. monocytogenes* strains expressing Mpl-Flag fusion proteins were grown in 250 ml of LB-MOPS-Glc, with chloramphenicol (10 mg/ml) if appropriate, to an OD_{600} ~ 0.8-0.9. The following protease inhibitors were added immediately after growth: phenylmethanesulphonylfluoride to 100 μM, leupeptin to 1 μM and pepstatin A to 1 μM (Sigma). The cultures were cooled on ice and all subsequent steps were performed at 4°C. Bacterial cultures were centrifuged at

10,000 rpm for 15 minutes. Supernatants were collected, filtered through a 0.22 μm PVDF membrane (Millipore), supplemented with sodium azide to 0.02%, and adjusted to pH 7.7 by the addition of NaOH. Supernatants were passed through 0.5 ml of mouse anti-flag M2 agarose beads (Sigma) pre-equilibrated with binding buffer (50 mM NaP, 300 mM NaCl, pH 7.7). The resin was washed 5 times with 5.0 ml of binding buffer including incubation for 5 minutes on a nutator for each wash. Antibody-bound protein molecules were eluted by the addition of 1.0 ml of 3X Flag peptide (Sigma) at 100 μg/ml in binding buffer including 10 minute incubation on a nutator. Two elutions were completed then combined. 50 μl of elution was analyzed for Mpl protein by Western immunoblot using anti-Flag M2 (Sigma) mouse monoclonal antibody at 1/5000 (0.2 μg/ml) followed by goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase at 1/25,000 (24 ng/ml) (Jackson ImmunoResearch Laboratories, Inc.). One ml of the elution was TCA precipitated as described above then analyzed for the presence of PC-PLC by Western immunoblot using a polyclonal rabbit anti-PC-PLC antibody at 1/5,000 followed by a goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (24 ng/ml) (Jackson ImmunoResearch Laboratories, Inc.).

Immunofluorescent Staining. Detection of bacteria-associated Mpl was performed by adapting a method developed for the detection of bacteria-associated PC-PLC (19). Hela cells were seeded on 18 X 18 mm glass coverslips at a concentration of 4×10^5 per 35 mm tissue culture dish, infected at an MOI of approximately 50 with either HEL-798 (Mpl-Flag), HEL-943 (MplΔpro-Flag) or NF-L943 (Mpl) for 1 hour, then washed with phosphate buffered saline (PBS, pH 7.1). At 1.5 hours post infection gentamicin was added (10 μg/ml). At 3 hours post infection coverslips were washed with PBS, fixed in acetone/methanol (1:1 v/v) for 2 minutes, and then washed with

PBS and Tris buffered saline pH 8.0 with 0.1% TritonX-100 (TBS-TX). Samples were treated with 700U of mutanolysin (Sigma) in sodium phosphate buffer pH 6.0 for 15 minutes at 37°C. Coverslips were washed with TBS-TX and blocked for 15 minutes with TBS-TX plus 10% bovine serum albumin. Samples were reacted with anti-Flag M2 mouse monoclonal antibody (Sigma) at 1/1000 (1.0 μg/ml) for 2 hours, then with donkey anti-mouse conjugated to FITC (Jackson ImmunoResearch Laboratories, Inc.) at 1/1000 (1.5 μg/ml) for 45 minutes. Samples were extensively washed with TBS-TX followed by TBS, air dried, and mounted with Prolong Gold Antifade with DAPI (Molecular Probes) onto glass slides. Images were acquired with a Olympus BX51 fluorescent microscope equipped with an Olympus DP70 digital camera and software.

RESULTS

The absence of the propeptide does not affect Mpl stability or production. The metalloprotease of *L. monocytogenes*, Mpl, is secreted as a 55 kDa zymogen that undergoes intramolecular autocatalysis, releasing a 20 kDa propeptide and a 35 kDa catalytic domain (5). In this study, our aim was to determine the function of the Mpl propeptide. Propeptides often serve as intramolecular chaperones, assisting in the folding of enzyme catalytic domains, and contributing to the stability and activity of their protein partner (30). To assess the role of the Mpl propeptide, we deleted its chromosomal sequence by allelic exchange. The corresponding mutant strain (*mpl*Δpro) synthesizes an Mpl molecule (MplΔpro) that is secreted as a mature enzyme upon cleavage of the signal sequence. This mutation was generated in two *L. monocytogenes* background strains: the clinical isolate 10403S (4) and its isogenic mutant NF-L943 (29). NF-L943 contains a point mutation in the positive

transcriptional regulator PrfA (PrfA G155S) causing the overexpression of PrfA-dependent genes, including *mpl* and *plcB*. NF-L943 derived mutants were used as a matter of convenience for non-tissue culture assays, whereas 10403S-derived mutants were used for most tissue culture assays as the expression of PrfA-dependent genes is upregulated intracellularly.

Stability of MplΔpro was assessed in broth culture. Secreted proteins were TCA precipitated from the supernatant of broth grown bacteria and Mpl was detected by Western immunoblot (Figure 3.1). Four species of Mpl are typically identified by Western immunoblot: a 55 kDa species corresponding to the proform or zymogen, the mature form which runs aberrantly as a protein of approximately 40 kDa, a degradation product of 32 kDa, and the 20 kDa propeptide (5) (Figure 3.1, lane 1). An Mpl species co-migrating with the mature form of Mpl was detected in the lane corresponding to the *mpl*Δpro mutant (Figure 3.1, lane 2), indicating that absence of the propeptide did not influence Mpl secretion or stability in broth grown bacteria. Providing the propeptide *in trans* did not affect the secretion and stability of mature Mpl by the *mpl*Δpro mutant strain (Figure 3.1, lane 3). These results indicated that the propeptide is dispensable for Mpl to reach a stable conformation.

The propeptide is not required for Mpl activity *in vitro*. Proteolytic activation of the broad-range phospholipase C, PC-PLC, is mediated by active Mpl (5, 18, 25). To determine if the propeptide is integral to the generation of active Mpl, PC-PLC activity was monitored by spotting bacterial strains onto egg-yolk agar (EYA). PC-PLC activity was detected around bacterial colonies as a zone of opacity resulting from phospholipid hydrolysis (Figure 3.2, panels 1 and 6). This surrounding zone of opacity was not detected in absence of PC-PLC or Mpl (Figure 3.2, panels 2, 5, 7, 11),

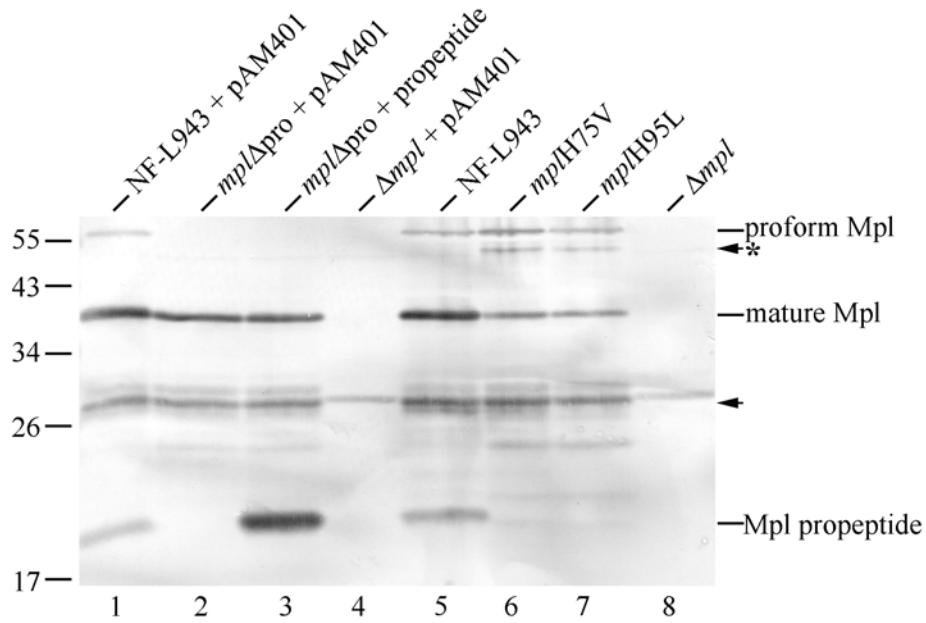


Figure 3.1. Detection of Mpl from culture supernatants by Western immunoblotting. Strains were grown in LB-MOPS-Glc, supplemented with chloramphenicol for strains carrying pAM401-derived plasmids. The equivalent of 1.0 ml of one OD_{600} was loaded into each lane. Mpl protein was detected using a rabbit anti-Mpl antibody. All strains are derived from NF-L943. Molecular weight markers in kDa are indicated on the far left. Positions of the proform, mature form and propeptide of Mpl are indicated at far right. Arrowhead indicates an Mpl degradation product consistently seen in broth grown cultures. Arrowhead with a star (*) indicates a novel degradation product from *mpl* H75V and *mpl*H95L. Mature Mpl is present in equivalent amounts from samples secreting wild-type Mpl, MplΔpro, and MplΔpro complemented with the propeptide *in trans.* The propeptide is present in samples secreting wild-type Mpl and MplΔpro complemented with the propeptide *in trans*, but absent from samples secreting MplΔpro, Mpl H75V, and Mpl H95L. Lanes: 1, Hel-940; 2, Hel-942; 3, Hel-938; 4, Hel-941; 5, NF-L943; 6, Hel-786; 7, Hel-782; 8, Hel-469. Experiment performed by B.M. Forster.

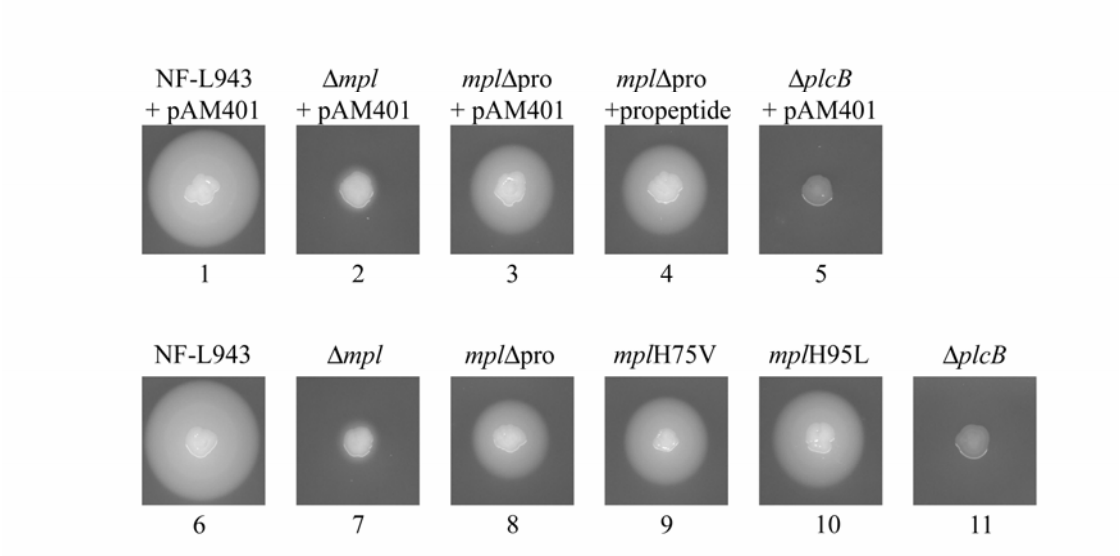


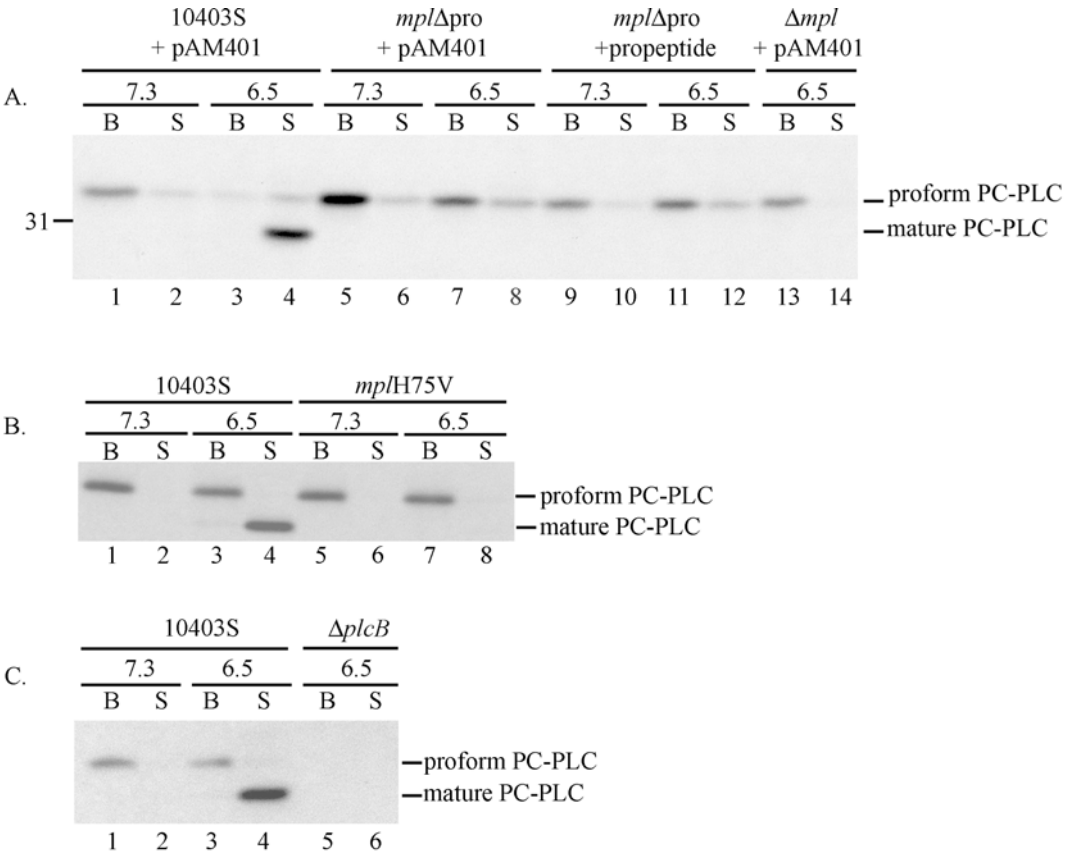
Figure 3.2. Detection of Mpl dependent PC-PLC activity on LB-egg yolk agar. Agar was inoculated with strains constructed in the NF-L943 background. Mpl-mediated activation of PC-PLC results in hydrolysis of egg yolk phospholipids creating a zone of opacity around the colony. Medium used for strains in panels 1-5 included chloramphenicol. Panels: 1, Hel-940; 2, Hel-941; 3, Hel-942; 4, Hel-938; 5, Hel-939; 6, NF-L943; 7, Hel-469; 8, Hel-927; 9, Hel-786; 10, Hel-782; 11, Hel-925.

confirming that the zone of opacity is generated by active PC-PLC and that activation of PC-PLC is Mpl-dependent. A zone of opacity was detected around *mpl*Δpro mutant colonies (Figure 3.2, panels 3 and 8) indicating that Mpl synthesized in absence of its propeptide is functional and capable of mediating PC-PLC maturation. Overall, these results demonstrated that the propeptide is not essential for Mpl to fold into an active form capable of mediating PC-PLC maturation.

The zones of opacity surrounding the *mpl*Δpro mutant colonies were smaller than the zones of opacity surrounding colonies expressing wild-type *mpl,* suggesting that the propeptide of Mpl contributes in part to Mpl activity. For the large majority of studied proteases, propeptides serve as intramolecular chaperones and are essential for their associated protease to reach native conformation. However, in many instances, native protein folding and protease activity can be rescued by providing the propeptide *in trans* (17, 20, 37, 45). Therefore, we sought to determine if providing the propeptide *in trans* could restore full Mpl activity. The sequence coding for the Mpl signal sequence and propeptide was cloned in a multicopy vector downstream of a constitutive spac promoter. As observed by Western immunoblot, the propeptide is secreted normally and appears stable when synthesized *in trans* of its protease partner (Figure 3.1, lane 3). On egg yolk agar, the zone of opacity surrounding the propeptide complemented *mpl*Δpro mutant strain was equivalent in size to the zone surrounding the non-complemented *mpl*Δpro mutant strain (Figure 3.2, panels 3 and 4). This result indicated that the defect observed with the *mpl*Δpro mutant cannot be complemented by providing the propeptide *in trans*. Overall these results indicated that the propeptide of Mpl is not essential to the generation of active Mpl. However, the propeptide enhances the production of active Mpl when present *in cis*, but not when provided *in trans*.

The propeptide is essential for intracellular Mpl activity. Mpl and PC-PLC contribute to the intracellular life cycle of *L. monocytogenes* by mediating bacterial escape from vacuoles. Intracellular bacteria maintain a pool of PC-PLC in its inactive proform. Rapid maturation and translocation of PC-PLC across the cell wall is dependent on Mpl activity and a decrease in pH (44). To assess if mature Mpl synthesized in absence of its propeptide is functional in a host cell environment, the behavior of PC-PLC was monitored in cells infected with the *mpl*Δpro mutant strain. Infected J774 cells were pulse-labeled with [^{35}S]methionine and the intracellular pH was manipulated during a chase period to mimic cytosolic or vacuolar pH. Infected cells were lyzed under conditions that preserve bacterial integrity, enabling physical separation of bacterial cells from host cell lysates, followed by lysis of the bacterial cells. The compartmentalization of PC-PLC was determined by immunoprecipitation of the protein from bacteria and host cell lysate fractions. As previously observed, PC-PLC remains primarily bacteria-associated and in its proform at pH 7.3 (Fig. 3.3A-C, lanes 1 and 2), whereas it is found primarily in the host cell fraction and in its mature form at pH 6.5 (Figure 3.3A-C, lanes 3 and 4). In the absence of Mpl, a decrease in pH does not influence PC-PLC compartmentalization as it remains bacteria-associated and in its proform at pH 6.5 (Figure 3.3A, lanes 13 and 14). The phenotype of the *mpl*Δpro mutant was similar to that of the Δ*mpl* mutant as PC-PLC remained primarily bacteria associated and in its proform upon a decrease in pH (Figure 3.3A, lanes 5-8). The defect of the *mpl*Δpro mutant could not be complemented by providing the propeptide *in trans* (Figure 3.3A, lanes 9-12). This result indicated that Mpl synthesized in absence of its propeptide *in cis* is unable to mediate the maturation and cell wall translocation of bacteria-associated PC-PLC upon a decrease in pH during intracellular infection, and synthesis of the propeptide *in trans* does not complement this defect.

Figure 3.3. Detection of Mpl mediated and pH dependent PC-PLC maturation and translocation across the bacterial cell wall in infected J774 cells. All strains are derivatives of 10403S. Infected cells were pulse-labeled with [^{35}S]methionine and chased in nigericin buffer adjusted to pH 7.3 or 6.5. Bacteria-associated (lanes labeled B) and secreted PC-PLC (lanes labeled S) were differentially immunoprecipitated from bacterial and host cell lysates respectively. PC-PLC was detected by autoradiography after protein fractionation by SDS-PAGE. Molecular weight marker in kDa is indicated on the far left. Intracellular pH (7.3 or 6.5) during the chase is indicated under strain names. Migration of proform and mature PC-PLC is indicated on the far right. A. Chloramphenicol maintained throughout experiment. The *mpl*Δpro mutant is unable to mediate PC-PLC maturation and translocation across the bacterial cell wall in response to a decrease in pH, even when the propeptide is provided *in trans*. Lanes: 1-4, Hel-903; 5-8, Hel-904; 9-12, Hel-908; 13-14, Hel-905. B. The *mpl*H75V point mutant is unable to mediate PC-PLC maturation and translocation across the bacterial cell wall in response to a decrease in pH. Lanes: 1-4, 10403S; 5-8, Hel-784. C. The Δ*plcB* strain serves as a negative control for the immunoprecipitation of PC-PLC proform and mature form. Lanes: 1-4, 10403S; 5-6, DP-L1935. Experiment in panel B performed by K. L. Roberts.


A.
10403S + pAM401
mplΔpro + pAM401
mplΔpro +propeptide
Δmpl + pAM401
7.3
6.5
B S B S B S B S B S B S B S
31
proform PC-PLC
mature PC-PLC
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B.
10403S
mplH75V
7.3
6.5
B S B S B S B S
proform PC-PLC
mature PC-PLC
1 2 3 4 5 6 7 8
C.
10403S
ΔplcB
7.3
6.5
6.5
B S B S B S
proform PC-PLC
mature PC-PLC
1 2 3 4 5 6

Mpl is also responsible for the pH-dependent cleavage of the bacterial surface protein ActA (26). Mpl-mediated cleavage of ActA was monitored as a secondary assessment of the intracellular activity of Mpl synthesized in absence of its propeptide. Infected J774 cells were pulse-labeled in the presence of host protein synthesis inhibitors, and the intracellular pH of the cells was manipulated during a chase period to mimic cytosolic or vacuolar pH as described above. ActA was extracted from the bacterial surface with sample buffer, resolved by SDS-PAGE and detected by autoradiography. ActA is phosphorylated during intracellular infection and forms a characteristic triplet on a protein gel (6) (Figure 3.4, lanes 1-3). As previously observed, membrane-associated ActA is lost upon a decrease in intracellular pH, and this phenomenon is dependent on Mpl activity (Figure 3.4, lanes 4 and 6). Bacteria-associated ActA was stable at acid pH in cells infected with the *mpl*Δpro mutant, similar to cells infected with the Δ*mpl* mutant (Figure 3.4, lanes 5 and 6). This result showed that the propeptide of Mpl is essential for Mpl-mediated cleavage of surface associated ActA. Overall, the results indicated that Mpl is unable to process its two known substrates, PC-PLC and ActA, during intracellular infection when it is synthesized in absence of its propeptide *in cis*, and providing the propeptide *in trans* cannot complement this defect.

The propeptide and catalytic domain of Mpl both interact with PC-PLC.

Following an observation that PC-PLC co-purifies with Mpl, we hypothesized that the propeptide of Mpl promotes Mpl and PC-PLC interaction. To test this hypothesis, we immunopurified mature Mpl-Flag from an *mpl*Δpro strain. Mpl-Flag and PC-PLC were detected by Western immunoblot. The ratio of eluate loaded on the protein gel was 1:20 for detection of Mpl and PC-PLC respectively. PC-PLC co-purified with Mpl synthesized in presence (wild-type Mpl) or absence (MplΔpro) of its propeptide

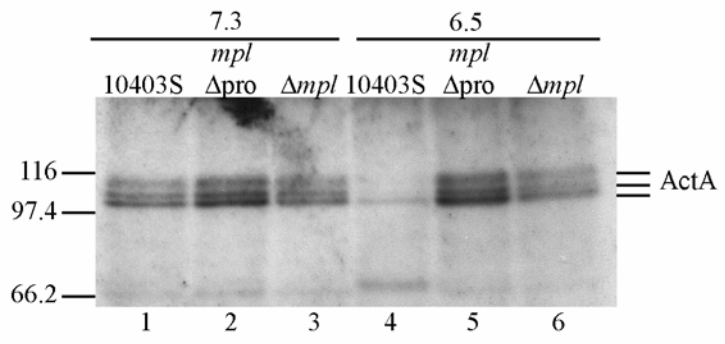
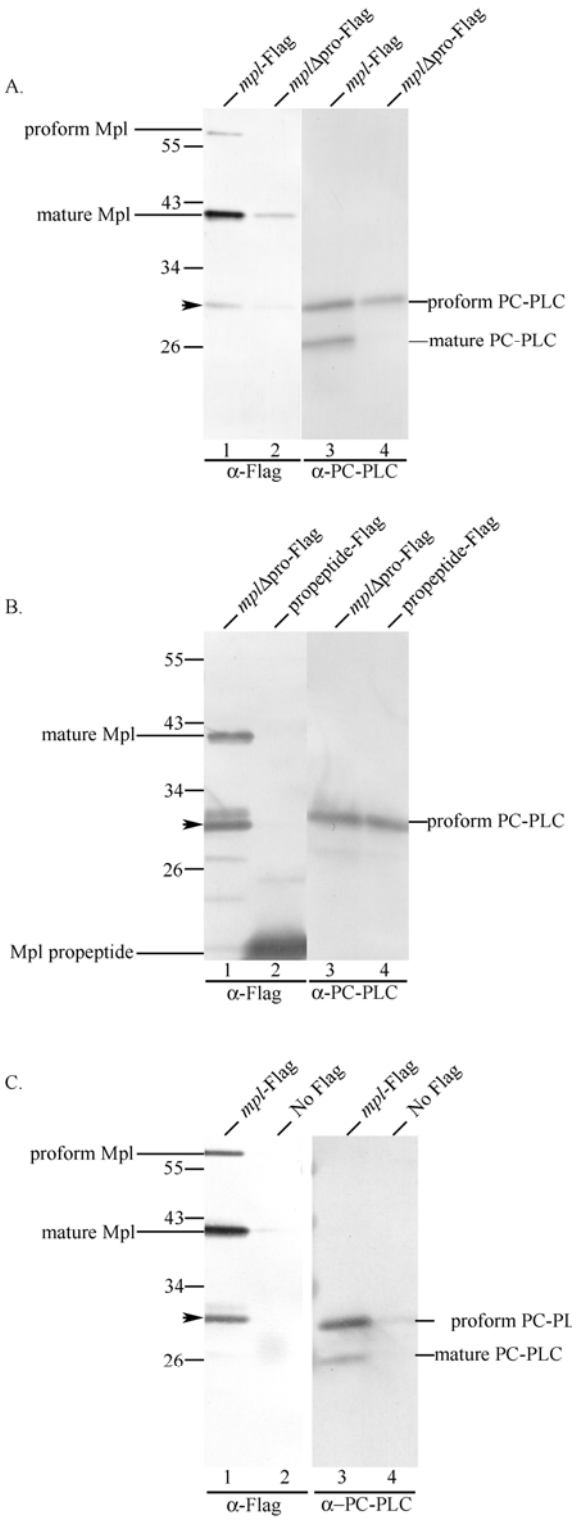


Figure 3.4. Detection of Mpl-mediated proteolytic cleavage of ActA during intracellular infection. J774 cells were infected with 10403S-derived strains. Cells were pulse-labeled with [^{35}S]methionine then chased with a nigericin containing medium at either pH 7.3 or 6.5. Molecular weight markers in kDa are indicated on the far left. Intracellular pH (7.3 or 6.5) during the chase is indicated over strain names. Migration of ActA triplet is indicated on the far right. A decrease in intracellular pH does not affect ActA stability in cells infected with Δ*mpl* or the *mpl*Δpro mutant strains as opposed to cells infected with wild-type strain. Lanes: 1 and 4, 10403S; 2 and 5, Hel-871; 3 and 6, DP-L2343.

(Figure 3.5A), indicating the propeptide is not required for Mpl to interact with PC-PLC. The ratio of Mpl to PC-PLC was equivalent between the two samples; however Mpl was present in higher concentration than PC-PLC within each sample. Next, we tested if the propeptide alone was able to interact with PC-PLC, by immunopurifying the Mpl propeptide with a C-terminal Flag tag from a Δ*mpl* strain. PC-PLC co-purified with the propeptide of Mpl (Figure 3.5B, lanes 2 and 4), indicating that the catalytic domain of Mpl is not essential for targeting Mpl to PC-PLC. However, the ratio of Mpl propeptide to PC-PLC was greater that the ratio of MplΔpro to PC-PLC, suggesting that the propeptide of Mpl has less affinity for PC-PLC than the catalytic domain. These interactions are not an artifact of PC-PLC non-specifically binding to the anti-Flag resin since PC-PLC did not bind to the resin when using a culture supernatant from a wild-type strain not expressing an Mpl-Flag protein (Figure 3.5C). These results demonstrated that the catalytic domain of Mpl has as much if not more affinity for PC-PLC than the Mpl propeptide. Therefore, the propeptide of Mpl is not required for Mpl to interact with PC-PLC.

The compartmentalization of Mpl is influenced by its propeptide during intracellular infection. During intracellular infection, the compartmentalization of PC-PLC is regulated in part by its propeptide. When synthesized with its propeptide, PC-PLC remains largely bacteria-associated during infection until the bacteria become entrapped in acidifying vacuoles. However, PC-PLC synthesized in absence of its propeptide is constitutively translocated across the bacterial cell wall in a manner independent of pH and Mpl (44). Therefore, we speculated that, similar to PC-PLC, the propeptide of Mpl influences its compartmentalization during intracellular infection. Results from immunofluorescence staining revealed the presence of bacteria-associated Mpl in cells infected with a strain of *L. monocytogenes* expressing

Figure 3.5. Co-immunoprecipitation of Mpl and PC-PLC from bacterial culture supernatants. Filtered supernatants from NF-L943-derived strains were used to immunoprecipitate Mpl-Flag constructs. 2.5% of the eluate was subjected to Western immunoblot using anti-Flag monoclonal antibodies for detection of Mpl. 50% of the eluate was subjected to Western immunoblot using a rabbit anti-PC-PLC polyclonal antibody. Mpl and PC-PLC species are indicated on the far left and right, respectively. Molecular weight markers in kDa are indicated on the left. Arrowhead indicates an Mpl degradation product. Antibody used for detection is indicated at the bottom. PC-PLC co-immunoprecipitated with wild-type Mpl-Flag (A), MplΔpro-Flag (A and B), and Mpl propeptide-Flag (B), but not in absence of an Mpl-Flag species (C). A. Lanes: 1 and 3, Hel-798; 2 and 4, Hel-943. B. Lanes: 1 and 3, Hel-943; 2 and 4, Hel-974. C. Lanes 1 and 3, Hel-798; 2 and 4, NF-L943.


A.
mpl-Flag
mplΔpro-Flag
mpl-Flag
mplΔpro-Flag
proform Mpl
55
43
mature Mpl
34
26
proform PC-PLC
mature PC-PLC
1 2 3 4
α-Flag
α-PC-PLC
B.
mplΔpro-Flag
propeptide-Flag
mplΔpro-Flag
propeptide-Flag
55
43
mature Mpl
34
26
proform PC-PLC
Mpl propeptide
1 2 3 4
α-Flag
α-PC-PLC
C.
mpl-Flag
No Flag
mpl-Flag
No Flag
proform Mpl
55
43
mature Mpl
34
26
proform PC-PL
mature PC-PLC
1 2 3 4
α-Flag
α–PC-PLC

Mpl-Flag (Figure 3.6, panel B). On the contrary, bacteria-associated Mpl was not detected in cells infected with the *mpl*Δpro-Flag or the strain expressing Mpl without a Flag tag (Figure 3.6, panels D and F). Detection of bacteria-associated Mpl was dependent on digesting the cell wall of fixed samples with mutanolysin (data not shown), indicating that bacteria-associated Mpl is not surface accessible, similar to bacteria-associated PC-PLC.

The influence of the Mpl propeptide on compartmentalization was also assessed by immunoprecipitating Mpl from cleared lysates of pulse-labeled infected cells. In wild-type infected cells, a small amount of the zymogen was inconsistently detected (Figure 3.7, lanes 1 and 5). In cells infected with the *mpl*Δpro mutant, the mature form of Mpl was consistently detected in larger amounts than the zymogen from wild-type infected cells (Figure 3.7, lanes 2 and 6). Complementation of the *mpl*Δpro strain with the propeptide *in trans* did not influence the behavior of MplΔpro (Figure 3.7, lane 3). Unfortunately, technical difficulties prevented us from immunoprecipitating bacteria-associated Mpl. Overall, these results suggested that intracellular bacteria maintain a pool of Mpl that is not surface accessible, and that Mpl is aberrantly secreted in the cytosol of infected cells when synthesized in absence of its propeptide.

During an investigation into potentially important propeptide residues, two point mutants with altered propeptide stability were discovered: *mpl* H75V and *mpl* H95L. These residues were originally targeted as possible pH sensors. Western immunoblot analysis of bacterial culture supernatants revealed the presence of a novel Mpl species below the proform around 48 kDa and the absence of the 20 kDa propeptide (Figure 3.1, lanes 6 and 7). Presumably, the 48 kDa band results from an initial cleavage within the propeptide, as the size of the mature form and 32 kDa degradation product

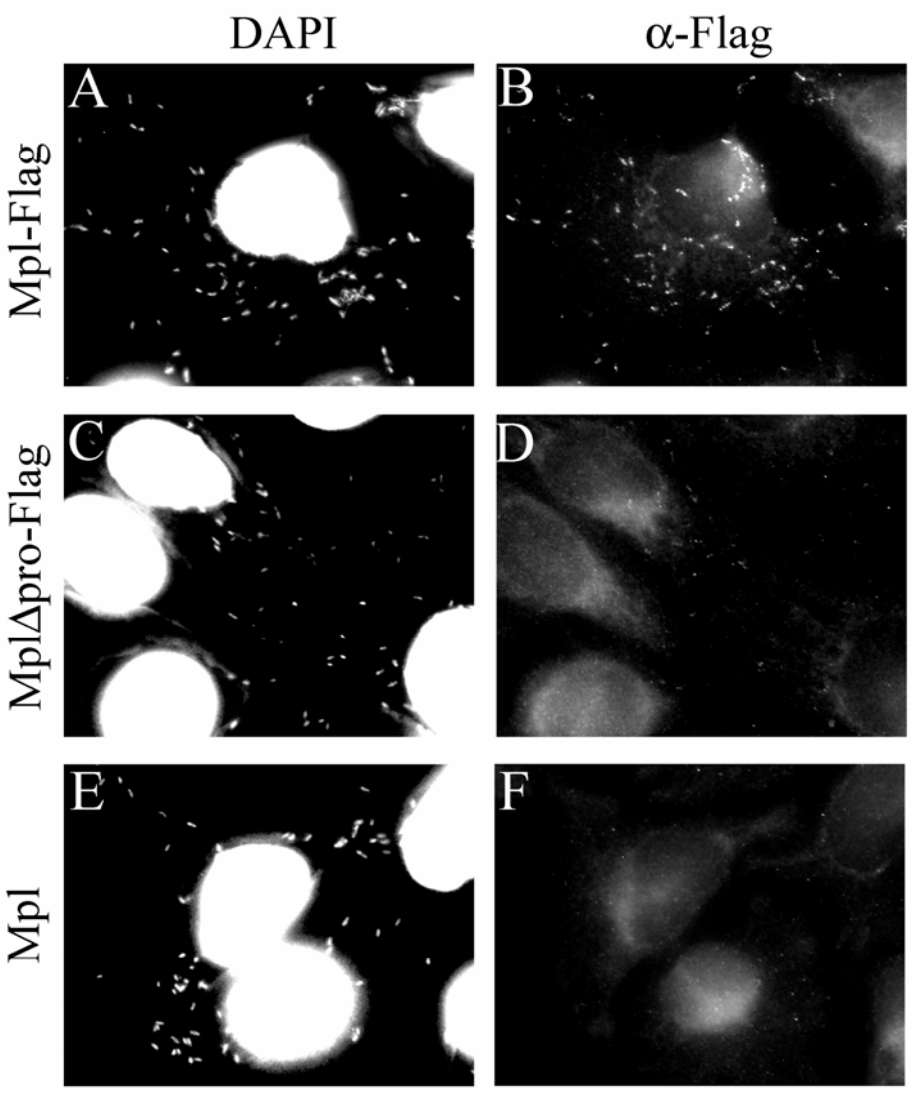


Figure 3.6. Detection of bacteria-associated Mpl in infected Hela cells by fluorescence microscopy. Hela cells were infected with NF-L943-derived strains. Fixed infected host cells were reacted with an anti-Flag antibody followed by a FITC conjugated secondary antibody. Bacteria and host cell nuclei were stained with DAPI. Mpl-Flag is detected as bacteria associated, whereas MplΔpro-Flag and Mpl lacking a Flag tag were not detected. A and B, Hel-798; C and D, Hel-943; E and F, NF-L943. Experiment performed by A.J. Chambers.

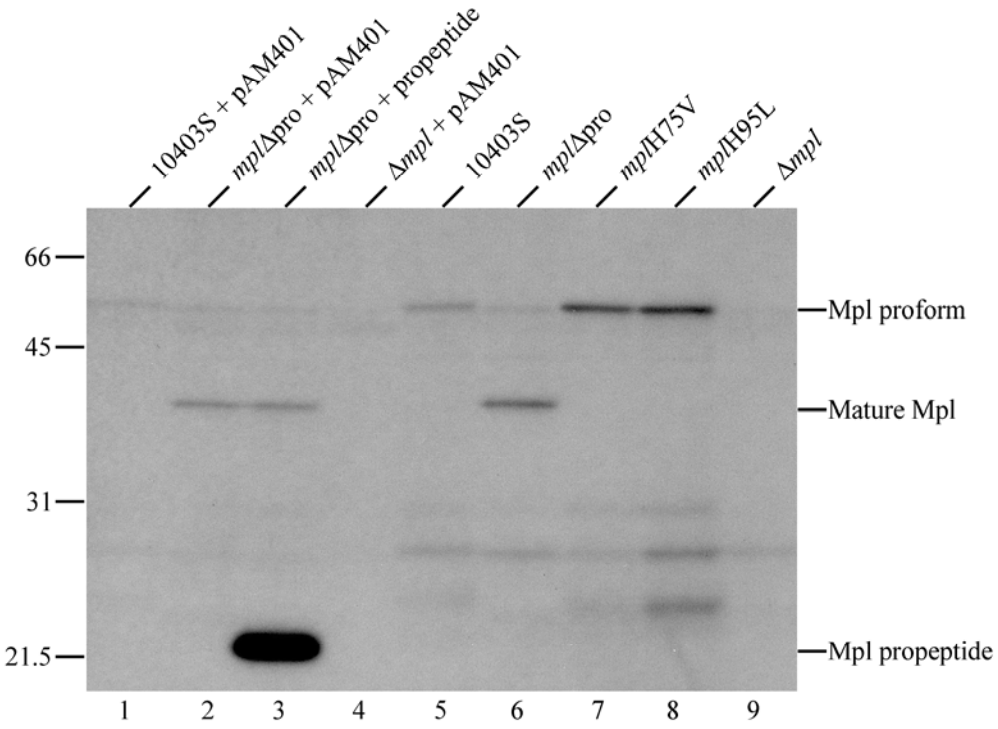


Figure 3.7. Immunoprecipitation of secreted Mpl from infected J774 cells. All strains are derivatives of 10403S. Infected cells were pulse-labeled with [^{35}S]methionine. Secreted Mpl was immunoprecipitated from cleared host cell lysates and detected by autoradiography after resolving the proteins by SDS-PAGE. Molecular weight markers in kDa are indicated on the far left. Mpl species are indicated on the far right. Samples from lanes 1-4 contained chloramphenicol throughout the experiment. The *mpl*Δpro and *mpl*Δpro + propeptide mutant strains show aberrant secretion of mature Mpl. The *mpl*H75V and *mpl*H95L mutant strains show aberrant secretion of the proform of Mpl. There is a non-specific band that comigrates with the proform of Mpl in the *mpl*Δpro lane. Lanes: 1, Hel-903; 2, Hel-904; 3, Hel-908; 4, Hel-905; 5, 10403S; 6, Hel-871; 7, Hel-784; 8, Hel-780; 9, DP-L2343. Experiment performed by B. M. Forster.

were unaltered, whereas the propeptide was unstable. This susceptibility to degradation suggests that the mutations affect the conformation of the propeptide. Mpl is not responsible for this novel cleavage, since when the H75V and H95L point mutations were constructed in a Mpl background that is catalytically inactive (E350Q) (5) the 48kDa product was still present (data not shown). The behavior of these two single site mutants was similar in many aspects to the behavior of the *mpl*Δpro mutant. On egg yolk agar, both single site mutants produced a zone of opacity slightly larger than the strain expressing MplΔpro but smaller than the zone produced by the wild-type strain (Figure 3.2, compare panels 9 and 10 to 8 and 6). In infected cells, neither *mpl* H75V nor *mpl* H95L mutant strains were able to mediate the maturation or translocation of PC-PLC in response to a decrease in pH, similar to the behavior of the *mpl*Δpro mutant strain (Figure 3.3B, lanes 5-8 and data not shown). The intracellular compartmentalization of Mpl was also altered in these two mutants. Immunoprecipitation of Mpl from cleared lysates of infected host cells revealed that the proform of Mpl H75V and of Mpl H95L was secreted in larger amounts than wild-type Mpl (Figure 3.7, compare lanes 7 and 8 to lane 5). This result suggested that misfolding of the propeptide, not its instability, causes the aberrant secretion of Mpl H75V and Mpl H95L. Together, these data indicated that the bacterial compartmentalization of Mpl is influenced by its propeptide during intracellular infection.

DISCUSSION

L. monocytogenes is a Gram-positive bacterial pathogen that multiplies in the cytosol of infected cells and spreads from cell-to-cell using an actin-based mechanism of motility (10, 39). Upon initial invasion of a host cell and during cell-to-cell spread, *L. monocytogenes* becomes entrapped in vacuoles which it must escape to perpetuate its

intracellular cycle. Among the factors contributing to bacterial escape from vacuoles are the phospholipase C, PC-PLC, and the metalloprotease, Mpl (31, 40). Both enzymes are made as proproteins, which undergo maturation by cleavage of a *N*-terminal propeptide. The propeptide of PC-PLC serves to inhibit enzymatic activity and to retard PC-PLC translocation across the bacterial cell wall (18, 19, 33, 44). Rapid maturation and translocation of PC-PLC across the cell wall depends on a decrease in pH and on Mpl activity. In this study, we sought to determine the function of the Mpl propeptide. Our results indicate that the propeptide of Mpl retains Mpl bacteria-associated, and that the compartmentalization of Mpl is integral to its ability to process its bacteria-associated substrates during intracellular infection.

Propeptides of bacterial proteases are normally important for catalyzing the folding of their covalently bound catalytic domains and can often perform this function either *in cis* or *in trans* (30). In this study, we first investigated whether the propeptide of Mpl serves as a folding catalyst by testing the behavior of a *L. monocytogenes* mutant lacking the sequence coding for the Mpl propeptide. Whether MplΔpro achieves native conformation was determined by assessing its enzymatic activity. Detection of Mpl from broth-grown bacteria by Western immunoblot indicated that the propeptide and catalytic domain fold into a stable form when synthesized as independent monomers. The EYA activity assay demonstrated that active Mpl can be generated in absence of the propeptide. Therefore, contrary to the large majority of studied proproteases, the propeptide of Mpl is dispensable for the production of active enzyme. This is not the first example of a bacterial metalloprotease whose propeptide is dispensable for activity as a similar observation was made for the neutral protease of *Bacillus stearothermophilus* (16). Additional observations led us to hypothesize that the propeptide of Mpl does not function as a folding catalyst. First, MplΔpro is less

active than wild-type Mpl in the EYA activity assay and this defect cannot be rescued by providing the propeptide *in trans*. Second, the propeptide appeared to be stable as it was detectable by Western immunoblot after cleavage from the proenzyme and when provided *in trans*. Together, these two observations suggested that the propeptide of Mpl does not interact with its associated protease *in trans*, since according to the folding catalyst model it would be degraded by its protease partner once native conformation is attained. Third, two destabilizing propeptide mutations, H75V and H95L, did not prevent the generation of an active mature form of Mpl. Alternatively, our observations could indicate that the propeptide of Mpl functions as a folding catalyst but that the free energy required for the transition from the intermediate to the native state is low, enabling the mature form to reach its native state in absence of the propeptide although with slower kinetics. This hypothesis would explain the decreased activity of MplΔpro detected on EYA, but would not explain the propeptide stability or why it cannot complement this defect *in trans*. Biochemical and biophysical analyses of purified Mpl will be required to solve whether the propeptide functions as a folding catalyst. However, our data clearly indicate that Mpl is an atypical thermolysin-like protease, as its propeptide is not required to generate active enzyme.

PC-PLC and Mpl are virulence factors that contribute to the intracellular life cycle of *L. monocytogenes*. Therefore, we assessed Mpl activity during intracellular infection. Mpl mediates the rapid maturation and translocation of bacteria-associated PC-PLC and cleaves membrane-anchored ActA in acid-pH vacuoles. These two functions were tested by manipulating the intracellular pH of infected cells to synchronize the reaction for all intracellular bacteria. Interestingly, the MplΔpro protein did not demonstrate activity toward either of its two substrates during intracellular infection.

In addition, the two propeptide single site mutants, Mpl H75V and Mpl H95L, were not active toward PC-PLC during intracellular infection. ActA proteolysis by Mpl was not assessed for these two mutants. Together, these data indicated that the propeptide of Mpl is integral to the intracellular function of Mpl.

In an attempt to determine why the propeptide of Mpl is essential for its function during intracellular infection, we first tested the hypothesis that the propeptide facilitates the interaction of Mpl with PC-PLC. Analysis of results from co-immunoprecipitation experiments suggested that wild-type Mpl, the propeptide by itself, and the catalytic domain by itself are all similarly efficient at interacting with PC-PLC in the bacterial supernatant. This result was consistent with those of the EYA activity assay showing that MplΔpro is capable of interacting with PC-PLC and mediating its maturation. However, we were surprised to observe that mature PC-PLC was not detectable in the supernatant of the MplΔpro broth grown bacteria since PC-PLC activity was detected on EYA. Perhaps, the mature form of PC-PLC has no affinity for the mature form of Mpl. Alternatively, these two proteins may be prevented from interacting with each other in broth because of the dilution factor, as opposed to the limited diffusion occurring on a semi-solid medium like the EYA. We concluded that the propeptide is not solely responsible for the interaction between Mpl and PC-PLC.

The behavior of PC-PLC during intracellular infection suggests that Mpl and PC-PLC interaction occurs prior to translocation across the bacterial cell wall, in the confined interface between cell wall and membrane. Perhaps, the propeptide of Mpl serves a function similar to the propeptide of PC-PLC, to retard translocation of the protein across the bacterial cell wall. To test this possibility, the compartmentalization of Mpl

during intracellular infection was determined. By immunofluorescence microscopy, we observed that wild-type Mpl, but not MplΔpro, associates with intracellular bacteria. Additionally, using an immunoprecipitation assay, we found MplΔpro in the secreted fraction from intracellular bacteria. Mpl H75V and Mpl H95L, which have an unstable propeptide in broth cultures, were also found in the secreted fraction of intracellular bacteria in larger amount than wild-type Mpl. Interestingly, the propeptide of these single site mutants appeared stable in infected cells, possibly because the protease responsible for the cleavage does not have access to Mpl or is inhibited. Together, the data support the hypothesis that the propeptide of Mpl serves a function similar to the propeptide of PC-PLC, to retard translocation of the protein across the bacterial cell wall.

In conclusion, this study revealed that the propeptide of Mpl serves to retain the protease bacteria-associated, and this function is integral to the ability of Mpl to mediate the rapid maturation and translocation of PC-PLC across the bacterial cell wall during intracellular infection. There are many similarities between Mpl and PC-PLC. Both enzymes are synthesized as a proprotein; both enzymes are found bacteria-associated during intracellular infection; their individual propeptides serve to retard protein diffusion across the bacterial cell wall; neither propeptide contain a transmembrane domain or a cell wall anchoring motif; and finally maturation of PC-PLC is dependent on Mpl activity. Future studies will aim to determine the mechanism by which these propeptides interfere with protein translocation across the bacterial cell wall, a phenomenon that is critical to the pathogenesis of *L. monocytogenes*.

Results from this study also revealed that the propeptide of Mpl is not required for Mpl to fold into its native state. However, additional biochemical and biophysical studies will be required to determine if the propeptide of Mpl can also function as a folding catalyst.

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CHAPTER 4

CONCLUSION

1. Brief summary of findings for chapter 2

This study examined the role of the flagella in host cell invasion. Previous work by our lab as well as Dons *et al*. (3) suggested that flagella may be used as adhesins. We generated a fully flagellated but non-motile mutant by introducing a point mutation in a flagella motor protein (*motB*D23A). This provided us with an excellent tool for assessing the importance of flagella. This mutant, a non-flagellated mutant and the wildtype were compared in tissue culture assays. Neither non-motile mutant was able to adhere to or invade cells as well as wildtype bacteria. In fact, the mutant with paralyzed flagella was 10 fold less adherent than the non-flagellated mutant. This same result was found with wildtype bacteria treated with sodium azide to prevent motility. This indicated that flagella are not used as adhesins. In fact, the presence of non-motile flagella was detrimental. Centrifugation of the non-motile mutants onto the host cell monolayer improved invasion, but not to wildtype levels. The improvement was greater though, for the non-flagellated mutant. This again suggested that the paralyzed flagella were actually impeding to invasion. The non-flagellated mutant was also used in the competitive infection of an oral mouse model. The loss of flagella and motility resulted in a 2.2 fold decrease in colonization of the mouse intestine and liver at early time points.

2. Future work for chapter 2

The creation of a non-motile but flagellated mutant proved to be a useful tool for differentiating between the role of the flagella as a potential adhesin and the role of flagella as a source of motility. This mutant was subsequently used in a study to examine the role of flagella in biofilm formation (9). The *motB*D23A mutant and a non-flagellated mutant were equally defective in adhesion to abiotic surfaces and biofilm formation, indicating the importance of flagella as a generator of motility and

not as adhesins. Biofilm formation is an important issue in control of *L. monocytogenes* in food production facilities.

The 2.2 fold defect of the non-flagellated mutant in the mouse model was smaller than we anticipated based on the much larger defect in tissue culture assays. Additionally, the centrifugation assay suggested that motility contributes more to invasion than simply increasing the probability of bacterial contact with host cells. We hypothesized that motility may be important for the quality of the interaction between bacteria and host cell. Perhaps bacteria need to approach host cells "head-on" to increase contacts between bacterial ligands that have a polar distribution, like InlA, and host cell receptors (8). An interesting experiment to conduct would be to determine the phenotype of the non-flagellated and flagellated but non-motile mutants in an animal model that was permissive to E-cadherin/InlA interactions. What would the invasion defect be in either a guinea pig or transgenic mouse? Another alternative would be to use a *L. monocytogenes* background with the InlA ligand mutated to bind to mouse E-cadherin (13). If the E-cadherin/InlA interaction is improved by motility then our mutants should show a greater virulence defect than 2.2 fold.

It would also be useful to confirm that flagella are present in the gut. There is data looking at flagella gene expression, but it is difficult to assess how this might apply to actual flagellar presence inside the host (5, 6, 11). However, bioluminescent imaging of mice infected with a strain of *L. monocytogenes* harboring the *lux* operon under the control of the flaA promoter clearly shows promoter activity throughout the entire infection process (7). Bacteria that are motile in the environment may maintain their flagella and have a competitive advantage over bacteria that are not motile once inside the host. Fluorescence microscopy to examine infected mouse intestines with

antibodies directed specifically at *L. monocytogenes* as well as flagella would help visualize the presence of flagella as well as their involvement in the interaction of the bacteria with the host cell.

3. Brief summary of findings for chapter 3

This study explored the function of the metalloprotease, Mpl, propeptide. Often propeptides are important for the folding of their covalently bound protein partners. We found that deletion of the propeptide from Mpl still generated active protein as indicated by its continued ability to mature PC-PLC *in vitro*. However, there was no indication of Mpl activity in multiple intracellular assays. We were able to demonstrate that Mpl synthesized without a propeptide was aberrantly secreted into the host cytosol. This loss of compartmentalization prevented Mpl from performing its intracellular functions.

We propose that Mpl is stored at the bacterial membrane / cell wall interface due to the presence of its propeptide (Figure 1.10). The deletion of the propeptide prevents the retention of the protein in this compartment, resulting in constitutive translocation across the cell wall. This eliminates Mpl's intracellular activity since once it has translocated across the cell wall it is unable to act upon its substrates, most likely due to host cell degradation of bacterial proteins or possibly because the conditions are no longer favorable for activity. This model helps explain the discrepancy between our *in vitro* egg yolk assay and our intracellular assays. The translocation of MplΔpro as well as the constant slow translocation of proform PC-PLC leads to a build up of both proteins in the egg yolk agar. Once in the agar MplΔpro is able to mature PC-PLC and PC-PLC can hydrolyze the phospholipids in the egg yolk. The ability of Mpl and PC-PLC to interact outside of the bacteria can be shown by streaking a strain that

express Mpl but not PC-PLC (Δ*mpl*) as well as a strain that expresses PC-PLC but not Mpl (Δ*plcB*). Where the two strains grow near to each other, Mpl and proform PC-PLC are able to diffuse and interact. This allows Mpl to mature PC-PLC and subsequently for PC-PLC to cause a zone of opacity in the agar between the two strains (Figure 4.1).

4. Future work for chapter 3

The immunoprecipitation of Mpl from infected host cells, as well as the immunofluorence staining of Mpl during tissue culture infection, demonstrated the inability of MplΔpro to remain bacterially associated. What is the mechanism that the propeptide uses to retain Mpl bacterially associated? Is there a conformation change once Mpl processes the propeptide, leading to a form that is more readily translocated across the cell wall? A comparison of crystal structures with and without the propeptide would be very useful. Of course this would require purified proteins. Purification of Mpl in large quantity has proven difficult but may be possible with further troubleshooting. Purified Mpl and MplΔpro could also be used in various biochemical assays such as circular dichroism or fluorescence spectroscopy to measure the kinetics of folding and unfolding in the presence or absence of the propeptide, although it is difficult to assess the applicability of these types of *in vitro* assays to *in vivo* conditions.

Additionally, it would be useful to quantitatively measure Mpl and MplΔpro activity, instead of the more qualitative measurement of PC-PLC activity on egg yolk agar. This can be done using an isotope based phospholipase assay (4). Another possible technique would be using purified Mpl and a substrate peptide in a fluorescence resonance energy transfer (FRET) assay. This involves using a peptide encoding a

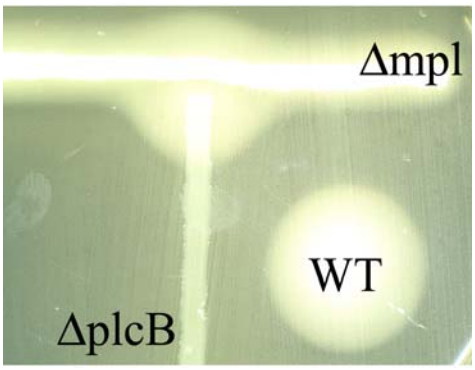

Figure 4.1. The interaction of Mpl and PC-PLC outside of bacteria in egg yolk agar. A strain (Hel-469) with a deletion in the Mpl gene (Δ*mpl*) is streaked horizontally and a strain (Hel-925) with a deletion in the PC-PLC gene (Δ*plcB*) is streaked vertically. Where the two strains grow close together but do not touch a zone of hydrolysis forms due to the maturation of PC-PLC by Mpl and the subsequent hydrolysis of phospholipids by mature PC-PLC.

cleavage site with a fluorescent molecule on one end and a quencher molecular on the other end. This quenched peptide would serve as a target for Mpl activity. If successfully cleaved fluorescence would increase. Initial experiments using a peptide that mimics the Mpl cleavage site have not been successful. However, our lab is improving the protocol to purify Mpl in a larger and cleaner quantity which may prove beneficial in this type of assay. FRET could also be tried with a peptide that mimics the PC-PLC cleavage site, the putative Mpl substrate.

Alternatively, there may be other proteins involved in the retention of Mpl and/or its interactions with PC-PLC. Since Mpl and PC-PLC compartmentalization can be different during *in vitro* growth and infection, it would be most informative to isolate these putative complexes during intracellular growth. This could be difficult since Mpl is not an abundant protein. Preliminary data suggests that if infection protocols are scaled up we can detect a sufficient amount of Mpl from the cell wall / membrane interface of bacteria in infected cells. Crosslinking proteins in this fraction before isolation would be one way to begin searching for protein partners of Mpl.

PC-PLC compartmentalization is dependent on pH (10, 12). Translocation of PC-PLC is dependent on Mpl and on pH, but is not dependent on PC-PLC processing (15). Is there also a relationship between Mpl and pH? Perhaps PC-PLCs dependence on pH is actually because Mpl requires a drop in pH in order to perform its translocation function? Is the autocatalytic processing of Mpl dependent on pH? It would be interesting to adapt the Mpl immunoprecipitation protocol to include the acidic nigericin buffer step similar to the PC-PLC immunoprecipitation experiment (10). Would Mpl be translocated dependent on pH? If so, what forms of Mpl would translocate?

We demonstrated that the absence of the propeptide affects Mpl retention. What would be the phenotype of an Mpl that could not be processed? The cleavage of Mpl occurs exclusively by intramolecular autocatalysis between glutamate 200 and valine 201 (1). Members of the thermolysin family, such as Mpl, tend to hydrolyze substrates with bulky, hydrophobic residues at the P1' position (2). An initial attempt to mutate the valine to an aspartic acid or a threonine resulted in an Mpl that was still able to mature. More extensive mutations would be required to generate a mutant with a cleavage site that could not longer be processed. This would provide an interesting look at what residues are permissive to cleavage and would help determine the requirements for Mpl proteolysis including any possible relationship to pH. Would proform Mpl remain bacteria associated at all times? What would be the effect of pH on this mutant? A drop in pH is a trigger for translocation of PC-PLC regardless of processing. Could this also be true for Mpl? This could also be investigated using the MplE350Q mutant, a catalytically dead mutant that cannot process itself (1). Does MplE350Q remain bacterially associated regardless of pH? Is Mpl's translocation dependent on activity?

The immunoprecipitation of Mpl from the bacteria associated fraction during intracellular infection was unsuccessful due to high background. However, the detection of bacteria associated Mpl during intracellular infection with immunofluorescence microscopy was able to show the presence of Mpl. Unfortunately this technique is unable to differentiate which forms of Mpl are present in this bacteria associated pool because of the antibodies used. The constitutive secretion phenotype of the MplΔpro mutant suggests that during infection with wildtype bacteria only the proform of Mpl would remain associated and that the processed form would be secreted. Our current Mpl antibody can cross react with

several other proteins and it is possible that an antibody with improved specificity might decrease background during immunoprecipitation. It would be useful to perform immunofluorescence on infected host cells with differential antibodies to determine which forms of the protein are retained. Addition of a nigericin pH manipulation step on the infected cells, similar to Marquis and Hager 2000 (10) could also be informative.

We have clearly shown the requirement of the propeptide for Mpl compartmentalization and activity in intracellular assays. The importance of compartmentalization of PC-PLC to virulence is well established (14). The next step would be to evaluate the virulence of the MplΔpro mutant. Will it have the same phenotype as a PC-PLC deletion mutant? What are the consequences of not degrading ActA? Both tissue culture assays, microscopy to examine vacuolar escape and actin polymerization, as well as an *in vivo* model are needed.

In summary, our data indicate a role for flagella mediated motility in host cell invasion and virulence. Our data also indicate the importance of the Mpl propeptide in intracellular function, and that presence of the propeptide is crucial for retaining Mpl bacteria associated. It appears that the use of the propeptide for protein compartmentalization is shared between Mpl and PC-PLC, two related virulence factors in *L. monocytogenes*.

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APPENDIX 1

1. Introduction

The compartmentalization of PC-PLC is critical *to L. monocytogenes* virulence (5). PC-PLC is stored as a pool of inactive proprotein at the bacterial membrane / cell wall interface (3, 4). Deletion of the propeptide from PC-PLC results in constant secretion of the active phospholipase regardless of pH or the presence of Mpl (6). A chimeric protein with the PC-PLC propeptide fused to the *B. cereus* PLC (PLC_{BC}) ortholog is translocates constantly (7). Bacteria expressing this chimera also show more efficient escape from the primary vacuole of tissue culture cells, a defect in cell to cell spread due to cytotoxicity, and an increased LD_{50} in an I.V. infection of mice (7). Therefore, the propeptide of PC-PLC is not sufficient for maintaining PLC_{BC} bacteria associated. Consequently, we decided to investigate if the catalytic domain of PC-PLC also played a part in the regulation of translocation. To determine the possible portion(s) of the PC-PLC catalytic domain required for compartmentalization several more chimeric proteins were constructed.

2. Chimera construction

A threading alignment of the *L. monocytogenes* PC-PLC and PLC_{BC} ortholog was performed by Hélène Marquis (Figure A1.1). There was a 41% identity and a 66% similarity between the proteins. Two areas of random coil were chosen as areas suitable for fusion (Figure A1.1, green and red highlighting). Chimeras were constructed using SOEing PCR and allelic exchange by Amanda J. Kreuder and Alan Pavinski Bitar. The chimeras constructed are schematically depicted in Figure A1.2.

3. Results of chimera characterization

To determine chimera protein stability, western immunoblot of culture supernatants

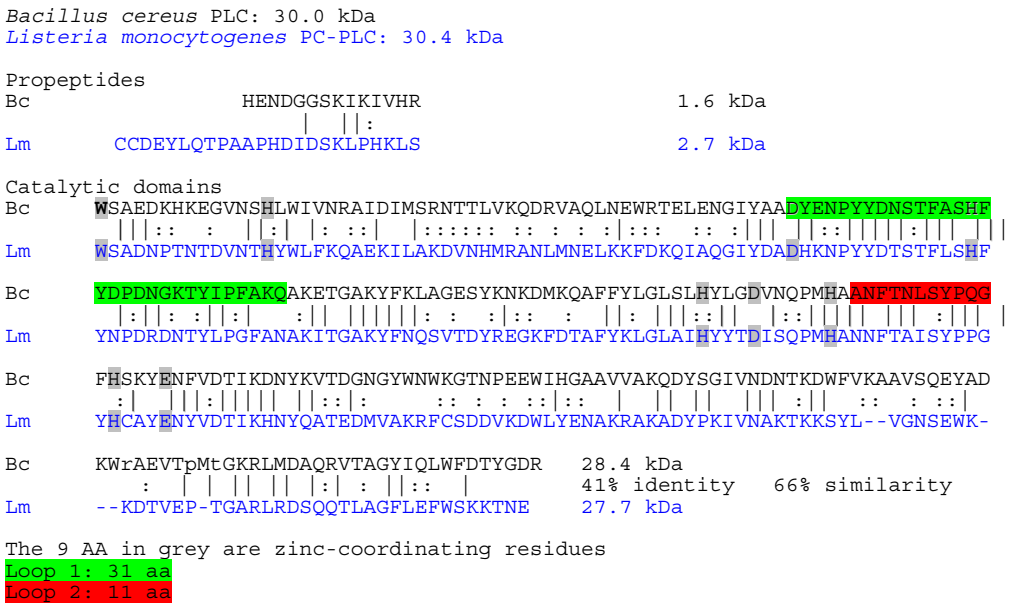

Figure A1.1. Threading alignment of *B. cereus* PLC and *L. monocytogenes* PC-PLC. Green and red highlighted areas indicate random loops where fusions were attempted between the two molecules. Gray boxes indicate zinc coordinating residues. Alignment performed by Hélène Marquis.

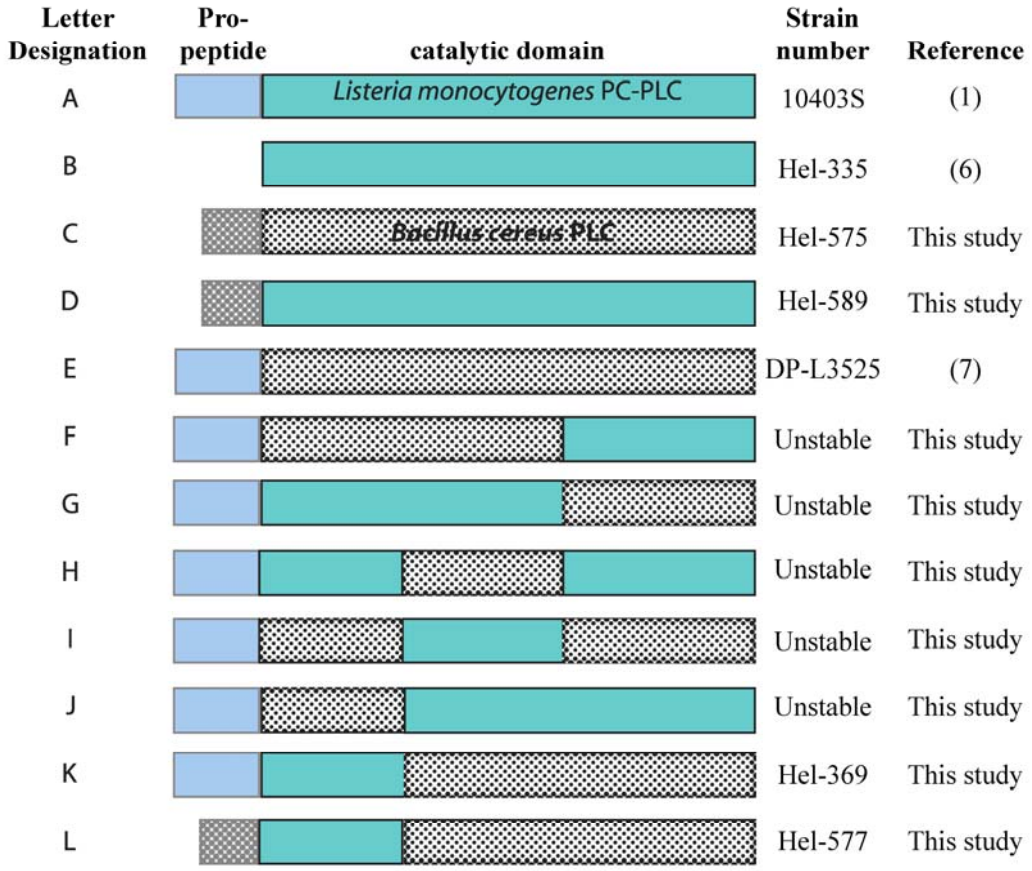

Figure A1.2. Schematic of the chimeras made with the *L. monocytogenes* PC-PLC and the *B. cereus* PLC ortholog. For ease of discussion each chimeric strain was given a letter designation, listed at far left. Strain number indicated on right along with reference. Schematic adapted from Hélène Marquis.

was performed by Alan Pavinski Bitar using previously described methods (Figure A1.3) (2). Briefly, cultures were grown under PrfA inducing conditions (Luria Broth with 50mM morpholinepropanesulfonic acid, adjusted to pH 7.3 and supplemented with 0.2% activated charcoal and 25mM glucose-1-phosphate at 37°C without shaking). Then proteins were TCA precipitated from culture supernatants and resolved on an SDS-PAGE gel, electrotransferred to polyvinylidene difluoride membrane and reacted with affinity-purified antibodies to both PC-PLC and PLC_{BC} (kindly provided by Howard Goldfine (7)) followed by alkaline phosphatase conjugated goat-anti-rabbit IgG. Unfortunately, chimeras F through J were unstable. Instability seemed to result from fusion at the second loop (Figure A1.1 red highlighting). However, chimera J was also unstable even though the fusion took place at the first loop. The instability of this chimera was surprising since chimera K was stable despite fusion at the same site. Yeuting Zhang deleted the propeptide from chimera J but it remained unstable.

As has been seen before, the wildtype 10403S strain generates two bands, the higher molecular weight band corresponds to the full length proform of PC-PLC, while the lower molecular weight band corresponds to the mature form after the propeptide has been cleaved (Figure A1.3). Chimera D, the *B. cereus* propeptide fused to *the L. monocytogenes* catalytic domain, produces a single band slightly higher than 28kDa. This must be the unprocessed proform, since processing would result in a band equal to the mature form of the wildtype. Chimera E, the *L. monocytogenes* propeptide fused to the *B. cereus* catalytic domain, shows two bands an unprocessed and a processed form that are slightly smaller than the wildtype species since the catalytic domain of PLC_{BC} is smaller than the catalytic domain of PC-PLC. Strain C, which has the entire PLC_{BC} protein, is unprocessed. If processing had occurred this band

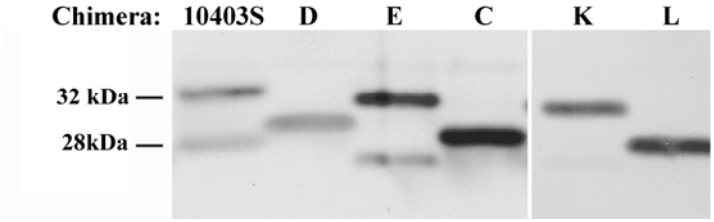

Figure A1.3. Detection of PLC chimeras from culture supernatants by western immunoblotting. Strains were grown in LB-MOPS-Glucose-1-phosphate then proteins were TCA precipitated from supernatants. The equivalent of 1.0 ml of one OD600 was loaded into each lane. PC-PLC protein was detected using rabbit anti-PC-PLC and anti-PLC_{BC} followed by goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase. Molecular weight markers are indicated at far left. Strains are indicated along top (see Figure App 1.2). Experiment performed by Alan Pavinski Bitar.

would equal the size of the processed chimera E. The lack of processing for chimera D and PLC_{BC} suggest that maturation is not occurring after the *B. cereus* propeptide even though the first three amino acids after the cleavage site in PC-PLC and PLC_{BC} are identical (Figure A1.1). Chimera K has the *L. monocytogenes* propeptide and the first third of the catalytic domain fused to the last two thirds of the *B. cereus* catalytic domain. This chimera produces a single band. Chimera L has the *B. cereus* propeptide and the last two thirds of the catalytic domain but with the first third of the *L. monocytogenes* catalytic domain. This chimera also produces a single band. It cannot be definitively stated whether the bands from chimeras K and L are the processed or unprocessed forms.

Alan Pavinski Bitar assessed phospholipase activity by inoculating egg yolk agar plates as described in chapter 3. Phospholipase activity hydrolyzes the egg yolk phospholipds and results in a zone of opacity around the colony (Figure A1.4). These strains are in a 10403S background and therefore do not show as strong a zone of opacity as the NF-L943 based strains in chapter 3. A 10403S strain with the complete deletion of the PC-PLC gene (Δ*plcB*) was used as a negative control (see chapter 3). PLC_{BC} (Strain C) and the fusion of the *L. monocygenes* propeptide to the *B. cereus* catalytic domain (chimera D) do not generate activity. This is expected since no processing was seen by western blot. Chimera E, the *L. monocytogenes* propeptide fused to the *B. cereus* catalytic domain shows very strong phospholipase activity. This is also true for chimera K, the *L. monocytogenes* propeptide and first third of the catalytic domain fused to the last two thirds of the *B. cereus* catalytic domain. This increase in activity over wildtype PC-PLC is because PLC_{BC} has a much higher specific activity then PC-PLC (7). Chimera L is similar to chimera K, except that is has the *B. cereus* propeptide. The activity of this protein on egg yolk is much lower

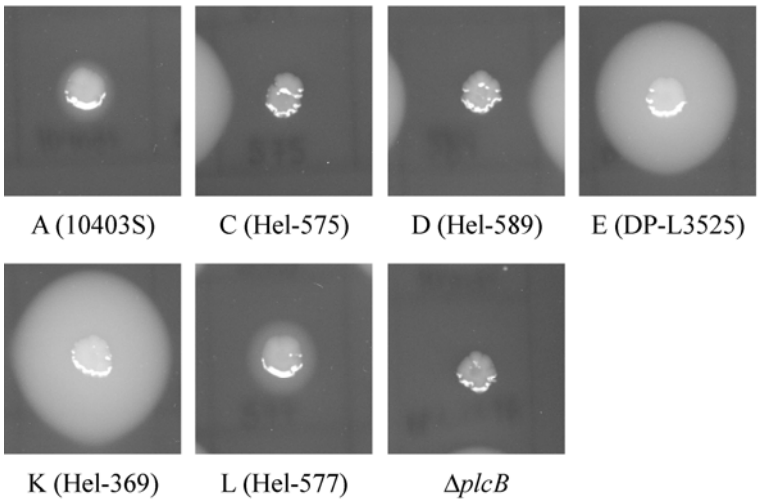

Figure A1.4. Detection of phospholipase activity on LB-egg yolk agar. Agar was inoculated and incubated for 48 hours. Phospholipase activity results in hydrolysis of egg yolk phospholipids creating a zone of opacity around the colony. The 10403S background strain (A) is shown in the first panel. The in-frame deletion of the PC-PLC gene serves as a negative control (last panel, Δ*plcB*). Experiment performed by Alan Pavinski Bitar.

than chimera K though. This is either because cleavage after the *B. cereus* propeptide is extremely inefficient and there is only a small amount of mature protein produced, or because the chimeric protein is not processed at all but still shows a small amount of activity.

To determine the pH dependent compartmentalization of the chimeras during intracellular infection, I performed immunoprecipitation assays using the methods described in chapter 3. Briefly, host cells were infected and pulse-labeled with [^{35}S]methionine and the intracellular pH was manipulated during a chase period to mimic cytosolic or vacuolar pH. Infected cells were lysed under conditions that preserve bacterial integrity, enabling physical separation of bacterial cells from host cell lysates, followed by lysis of the bacterial cells. The compartmentalization of PC-PLC and of the chimeras was determined by immunoprecipitation of the protein from bacteria and host cell lysate fractions. To ensure full immunoprecipitation of chimeric proteins, the protocol also included the antibody to the *B. cereus* PLC protein at a dilution of 1/40 (7). Again, the strain with a complete deletion of *plcB* was used as a negative control.

As previously observed, PC-PLC is primarily bacteria-associated and in the proform at pH 7.3 whereas it is primarily found in the host cell fraction and in the mature form at pH 6.5 (Figure A1.5). The addition of the *L. monocytogenes* propeptide to the *B. cereus* PLC is not sufficient to confer compartmentalization, as can be seen by the secretion of chimera E at pH 7.3. This chimera does show pH dependent cleavage of the propeptide. The opposite construct, chimera D, has the *B. cereus* propeptide fused to the *L. monocytogenes* PLC. This is not processed or compartmentalized properly and is secreted at pH 7.3. Apparently both the propeptide and the catalytic domain

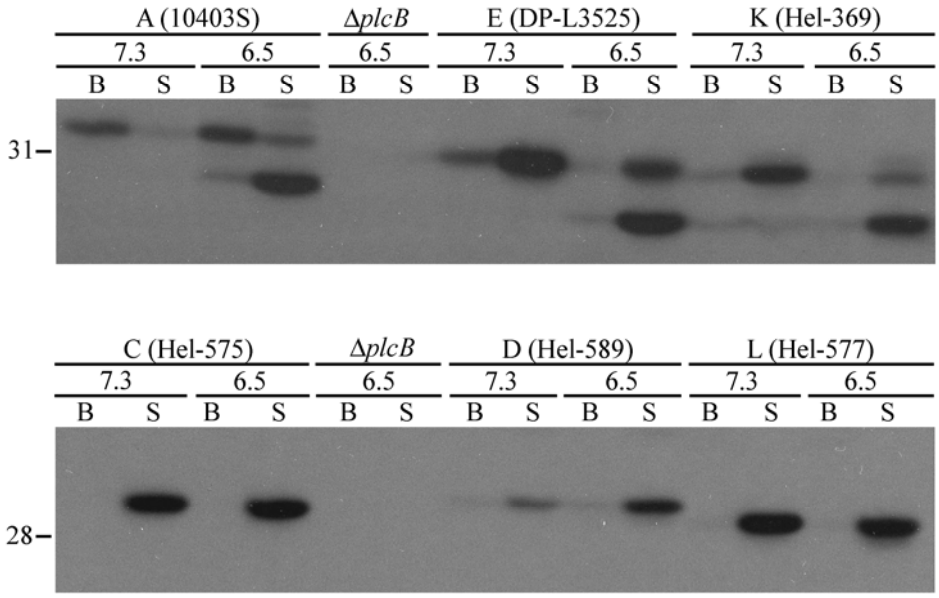

Figure A1.5. Detection of pH dependent PLC chimera maturation and translocation across the bacterial cell wall in infected J774 cells. Cells were pulse-labeled with [^{35}S]methionine and chased in nigericin buffer adjusted to pH 7.3 or 6.5, as indicated under strain. Bacteria-associated (labeled B) or secreted (labeled S) PC-PLC and chimeric PLC proteins were differentially immunoprecipitated from bacterial and host cell lysates respectively using antibodies to both PC-PLC and PLC_{BC}, then detected by autoradiography after protein fractionation by SDS-PAGE. Molecular weight marker in kDa is indicated on the far left and strains across top (see Figure A1.1).

participate in controlling the secretion of PC-PLC. Chimeras K and L contain the first third of the *L. monocytogenes* PLC and either the *B. cereus* or the *L. monocytogenes* propeptide. Neither protein shows pH dependent secretion. Chimera K does show pH dependent maturation. Chimera K indicates that while both the catalytic domain and the propeptide of *L. monocytogenes* are necessary for compartmentalization, the propeptide and the first third of the catalytic domain is not sufficient to confer proper compartmentalization to the protein. It is not clear if the bands for chimera L are the unprocessed or processed form. Strain C with the entire *B. cereus* PLC does not show pH dependent secretion or processing, as is expected.

The compartmentalization of PC-PLC requires the *L. monocytogenes* propeptide, but the addition of this propeptide to an orthologous protein is not sufficient to maintain the chimeric protein bacterial associated. Therefore, the PC-PLC catalytic domain also plays a role in this compartmentalization. The first third of the catalytic domain is not sufficient though, indicating that some part of the later two thirds of the catalytic domain is also required. Additional chimeras would need to be studied to determine what other portion of the catalytic domain controls compartmentalization along with the propeptide.

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APPENDIX 2

Table A2.1. Tissue culture cell lines

Cell Line	Organism	Origin	Morphology
Caco-2	Human	Colorectal adenocarcinoma	Epithelial
GPC-16	Guinea pig	Colorectal adenocarcinoma	Epithelial
HCT-8	Human	Ileocecal colorectal adenocarcinoma	Epithelial
HeLa	Human	Cervix adenocarinoma	Epithelial
Henle-407 (aka INT-407)	Human	Jejunum-ileum adenocarinoma	Epithelial
HEp-2	Human	Larynx adenocarcinoma	epithelial
HT-29	Human	Colorectal adenocarcinoma	Epithelial
HuTu-80	Human	Duodenum adenocarcinoma	Epithelial
J774	Mouse (BALB/cN)	Reticulum cell sarcoma	Macrophage
L2071	Mouse		Fibroblast
Vero	African green monkey	Kidney	Epithelial
WS-1	Human (fetal)	Kidney	Epithelail